

Abstracts of papers presented
at the 1995 meeting on

THE CYTOSKELETON & CELL FUNCTION

April 26–April 30, 1995



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13. ABSTRACT (Maximum 200 words) The Cytoskeleton and Cell Function meeting brought together scientists working on the cytoskeleton in invertebrate and vertebrate systems. Although much information is now available concerning the structural and molecular bases for the various cytoskeletal components, it is only recently that the function and regulation of these components in cellular processes is beginning to be understood. The meeting highlighted topics in actin filament assembly and dynamics, cell motility, microtubule assembly and dynamics, cell division, intermediate filaments, molecular motors, intracellular trafficking, signal transduction, membrane-cytoskeleton interactions, and development and differentiation. Various experimental approaches were presented, including biochemistry, genetics, and molecular biology. The sessions encompassed the most recent advances in the field and made for an intense and stimulating exchange of information.				
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Arranged by
David Helfman, *Cold Spring Harbor Laboratory*
Elizabeth Raff, *Indiana University*

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Front Cover: (Upper panel) Labeling of microfilaments in rat fibroblasts by microinjection of rhodamine-labeled recombinant rat fibroblast tropomyosin TM-5 demonstrating incorporation into stress fibers and at the leading lamella. *(Lower panel)* The same cells stained with FITC-phalloidin. (Courtesy of Connie Temm-Grove and Wei Guo, Cold Spring Harbor Laboratory.)

Back Cover: Triple immunofluorescent labeling of the cytoskeleton in fibroblasts using rhodamine-, fluorescein-, and coumarin-conjugated probes, as indicated. Actin is labeled with phalloidin. (Courtesy of Vic Small, Institute of Molecular Biology, Salzburg, Austria.)

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PROGRAM

WEDNESDAY, April 26—7:30 PM

SESSION 1 ROLE OF THE CYTOSKELETON IN DEVELOPMENT AND DIFFERENTIATION

Chairperson: L. Cooley, Yale University Medical School

Tilney, L.G., Tilney, M.S., Dept. of Biology, University of Pennsylvania,
Philadelphia: Actin bundles in *Drosophila* bristles—Two filament
crossbridges are involved in bundling. 1

Theurkauf, W.,¹ Wang, S.,² Hazelrigg, T.,² ¹Dept. of Biochemistry and
Cell Biology, State University of New York, Stony Brook; ²Dept. of
Biology, Columbia University, New York, New York: Direct
visualization of intercellular transport and anterior patterning during
Drosophila oogenesis. 2

Manseau, L., Phan, H., Dept. of Molecular and Cellular Biology,
University of Arizona, Tucson: Profilin is required to repress
microtubule-based cytoplasmic streaming within the *Drosophila*
oocyte. 3

Cooley, L., Cant, K., Mahajan-Miklos, S., Knowles, B., Matova, N.,
Dept. of Genetics, Yale University Medical School, New Haven,
Connecticut: Genetic analysis of actin dynamics during *Drosophila*
oogenesis. 4

Mermall, V., Miller, K.G., Dept. of Biology, Washington University, St.
Louis, Missouri: The *Drosophila* 95F unconventional myosin is a
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blastoderm embryo. 5

Chant, J., Stowers, L., Mischke, M., Freedman, T., MCB, Harvard
University, Cambridge, Massachusetts: The orientation and
formation of axes of cytoskeletal polarity. 6

Mangan, M.E., Olmsted, J.B., Dept. of Biology, University of
Rochester, New York: A muscle-specific variant of microtubule-
associated protein 4 (MAP 4) is expressed early in myogenesis. 7

Perriard, J.-C., Rothen-Rutishauser, B., Bantle, S., Haussmann, I.U., Komiyama, M., Mühlebach, S., Institute for Cell Biology, ETH, Hönggerberg, Zürich, Switzerland: Cytoskeletal dynamics and biogenesis of cytoarchitecture in cardiomyocytes. 8

Fischman, D.A.,¹ Gilbert, R.,¹ Alyonycheva, T.,¹ Lebedeva, M.,¹ Mikawa, T.,¹ Whittle, M.R.,² Reinach, F.C.,^{1,2} ¹Dept. of Cell Biology and Anatomy, Cornell University Medical College, New York, New York; ²Dept. of Biochemistry, University of Sao Paulo, Brazil: β barrels, myosin-binding proteins, and sarcomere assembly. 9

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Chairperson: **P.A. Rubenstein**, University of Iowa College of Medicine

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Marchand, J.-B.,¹ Moreau, P.,¹ Paoletti, A.,² Cossart, P.,³ Carlier, M.-F.,¹ Pantaloni, D.,¹ ¹Laboratoire d'Enzymologie, ²Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, ³Institut Pasteur, Paris, France: Actin-based movement of *Listeria monocytogenes* in *Xenopus* egg extracts is due to local uncapping of the barbed ends of actin filaments at the bacterium surface and resulting shift in steady state of actin assembly. 13

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THURSDAY, April 27—4:30 PM

Wine and Cheese Party

THURSDAY, April 27—7:30 PM

SESSION 4 MICROTUBULE ASSEMBLY AND DYNAMICS

Chairperson: E.C. Raff, Indiana University

- Raff, E.C., Fackenthal, J.D., Hoyle, H.D., Hutchens, J.A., Turner, F.R., Dept. of Biology, Indiana University, Bloomington, Indiana: Genetic analysis of tubulin requirements for axoneme assembly in *D. melanogaster*. 87
- Linck, R.W.,¹ Amos, L.A.,² Egelman, E.H.,¹ Nojima, D.,¹ Norrander, J.M.,¹ Pirner, M.A.,¹ ¹Dept. of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis; ²Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom: Molecular and structural analysis of tektins in flagellar microtubules. 88
- Gaertig, J.,^{1,3} Gu, L.,¹ Pennock, D.,² Gorovsky, M.,¹ ¹Dept. of Biology, University of Rochester, New York; ²Dept. of Zoology, Miami University, Oxford, Ohio; ³Dept. of Zoology, University of Georgia, Athens: Analysis of tubulin gene function using gene replacement in *Tetrahymena*. 89
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- Rao, S., Haber, M., Orr, G.A., Horwitz, S.B., Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York: Mapping of the taxol-binding site on tubulin—A photoaffinity labeling approach. 91
- Zhang, Y., Coffey, R.L., Green, C.L., Ainsztein, A.M., Purich, D.L., Dept. of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville: Microtubule and PKC interactions with bovine MAP-2 MT-binding region and 3-/4- repeat rat MAP-2C. 92
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Shuster, C.B.,¹ Herman, I.M.,^{1,2} ¹Program in Cell, Molecular and Developmental Biology, ²Cellular and Molecular Physiology, Tufts University Health Science Schools, Boston, Massachusetts: Indirect association of ezrin with β actin—Isoform specificity and calcium sensitivity. 197

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- Verkhovsky, A.B., Svitkina, T.M., Borisy, G.G., Laboratory of Molecular Biology, University of Wisconsin, Madison: Condensation of myosin II minifilament assemblies during cell protrusion. 199
- Simon, V.R., Swayne, T.C., Pon, L.A., Dept. of Anatomy and Cell Biology, Columbia University, College of Physicians & Surgeons, New York, New York: Role of the actin cytoskeleton in regulated mitochondrial movements during yeast cell division. 200
- Gunning, P.,^{1,3} Ferguson, V.,¹ Jacobsen-Lyon, K.,^{2,3} Brennan, K.,² Elsom, V.,² Hardeman, E.,^{2,3} ¹Cell Biology Unit, ²Muscle Development Unit, ³CRC for Cardiac Technology, Children's Medical Research Institute, Wentworthville, Australia: Isoform-specific communication between actin and tropomyosin multigene families. 201

SATURDAY, April 29—6:00 PM

Concert for Cello and Piano

Performed by

Alexis Gerlach, cello
and
Melvin Chen, piano

Program

Twelve Variations on a Theme from Handel's Oratorio,
Ludwig van Beethoven "Judas Maccabaus"

Suite No. 6 in D Major for Solo Cello, BWV 1012 J.S. Bach
Prelude
Allemande
Courante
Sarabande
Gavottes I and II
Gigue

From *Miroirs*, Suite for Solo Piano Maurice Ravel
Une barque sur l'océan
Alborada del Gracioso

Hungarian Rhapsody, Op. 68 David Popper

Concert sponsored by Roger H. Samet

SATURDAY, April 29

BANQUET

Cocktails 7:00 PM Dinner 7:45 PM

DANCE

Bush Auditorium 9:30 PM

SUNDAY, April 30—9:00 AM

SESSION 10 SIGNAL TRANSDUCTION:
MEMBRANE-CYTOSKELETON INTERACTIONS

Chairperson: **B. Geiger**, Weizmann Institute of Science, Rehovot,
Israel

Nobes, C.D., Hall, A., CRC Oncogene and Signal Transduction
Group, MRC Laboratory for Molecular Cell Biology, University
College London, United Kingdom: Rho, rac, and cdc42 GTPases
regulate the assembly of plasma membrane focal complexes
associated with three distinct types of actin structure. 202

Altun-Gultekin, Z., Stohl, L.L., Wagner, J.A., Dept. of Neuroscience,
Cornell University Medical College, New York, New York: Roles of
SRC, RAS, and RAC in the morphological changes observed in
migrating PC12 cells. 203

Bershadsky, A., Geiger, B., Dept. Chemistry and Immunology,
Weizmann Institute of Science, Rehovot, Israel: Activation of
adhesion-dependent signal transduction via cytoskeletal
perturbation—Formation of focal adhesions and actin bundles,
tyrosine phosphorylation of *FAK* and paxillin, and induction of DNA
synthesis in serum-starved cells after microtubule disruption. 204

Johnson, R.P., Craig, S.W., Dept. of Biological Chemistry, Johns
Hopkins University School of Medicine, Baltimore, Maryland: The
intramolecular association of vinculin head and tail domains—A
potential mechanism for regulating adherens junction assembly. 205

- Carraway, C.A.C., Carvajal, M.E., Lorenzo, D., Li, Y., University of Miami School of Medicine, Florida: A stable microfilament-associated signal transduction particle in mammary tumor microvilli—Presence of p185^{neu}, MAP kinase pathway components and E-cadherin/catenins. 206
- Lin, S., Lin, D.C., Dept. of Biophysics, Johns Hopkins University, Baltimore, Maryland: Identification of actin, vinculin, and integrin binding domains in tensin suggests new mechanisms for actin-membrane association. 207
- Nakamura, F., Amieva, M.R., Furthmayr, H., Laboratory of Experimental Oncology, Dept. of Pathology, Stanford University Medical Center, California: Site-specific phosphorylation and localization of moesin in thrombin-activated human platelets. 208
- Strand, D., Merdes, G., Neumann, B., Mechler, B.M., Dept. of Developmental Genetics, German Cancer Research Center, Heidelberg, Germany: Interactions between the *Drosophila lethal(2)giant larvae* tumor suppressor protein, p127, and nonmuscle myosin II heavy chain. 209
- Simcha, I.,¹ Geiger, B.,² Yehuda, S.,² Ben-Ze'ev, A.,¹ Depts. of ¹Molecular Genetics and Virology, ²Chemical Immunology, Weizmann Institute of Science, Rehovot, Israel: The cytoskeletal plaque protein plakoglobin suppresses the tumorigenicity of cells from mouse and human origin. 210

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ACTIN BUNDLES IN *DROSOPHILA* BRISTLES: TWO FILAMENT CROSSBRIDGES ARE INVOLVED IN BUNDLING.

Lewis G. Tilney and Mary S. Tilney, Dept. of Biology, University of Pennsylvania, Philadelphia, PA 19104.

Transverse sections through bristles reveal 7-11 nearly round plasma membrane associated bundles of actin filaments. These filaments are hexagonally packed and in longitudinal section show a 12 nm periodicity in both the 1,1 and 1,0 views. This periodicity is attributable to a crossbridge. From earlier studies on stereocilia, microvilli, and *Mytilus* sperm, this means that the filaments are maximally crossbridged. The singed mutants, Sn^3 or X^2 , also have 7-11 bundles, but the bundles are smaller, flattened, and the filaments within the bundles are randomly packed (not hexagonal) and no periodicity can be detected in longitudinal sections. This mutant lacks the actin crossbridge, fascin (Cant et al., 1994; JCB 125:369). Another mutant, forked, also has 7-11 bundles but even though the bundles are very small, 1/50 the size of the wild type, the filaments within them are hexagonally packed and display a 12 nm periodicity in the 1,0 and 1,1 views of longitudinal section. This mutant lacks a series of related proteins located by antibodies to the actin bundles in the wild type (Petersen et al., 1994; Genetics 136:173). Hints of why two species of crossbridges are necessary can be gleaned by studying bristle formation. Bristles sprout with only microtubules in them. A little later in development actin filaments are found near the plasma membrane and with time these come together to form bundles. The filaments in the bundles at early stages are randomly packed and lack the periodicity in longitudinal section. Later the filaments in the bundles become hexagonally packed and maximally crossbridged. Thus the forked proteins seem to be necessary early in development to tie the filaments together in a bundle so that they can be subsequently zippered together by fascin (the singed gene). This conclusion is strengthened by the observation that forked proteins appear in large concentrations early in development (Petersen, 1994; Mol. Biol. Cell 5s:273a).

DIRECT VISUALIZATION OF INTERCELLULAR TRANSPORT AND ANTERIOR PATTERNING DURING *DROSOPHILA* OOGENESIS

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Drosophila oogenesis is initiated by four incomplete mitotic divisions that produce cysts of 16 cells that are interconnected by cytoplasmic bridges. Only one of these 16 cells forms an oocyte, while the 15 remaining cells form nurse cells that synthesize maternal components that are transported to the oocyte. Specification of the anterior pole of the embryo requires transport of the bicoid mRNA anterior morphogen from the nurse cells to the single cell that will form the oocyte, and localization of this mRNA to the anterior pole of the oocyte. The *exu* gene is required for transport and anterior localization of bicoid mRNA. Previous studies demonstrate that a GFP-EXU fusion protein functions in anterior patterning and forms particles that accumulate in a microtubule-dependent manner at the ring canals that link the germline cells within egg chambers. We have used animals expressing this fusion protein and time-lapse confocal microscopy to directly visualize intercellular transport and anterior patterning during stages 7 through 9 of oogenesis. Experiments utilizing cytoskeletal assembly inhibitors indicate that the accumulation of GFP-EXU particles at ring canals is dependent on a relatively unstable population of microtubules, and that transport through the ring canals may depend on a more stable subset of microtubules. Actin filaments, in contrast, do not appear to be involved in GFP-EXU particle transport. These *in vivo* studies also show that particle transport through the ring canals that link the nurse cells and the oocyte is uniformly toward the oocyte, but that particles shuttle in both directions through more distal ring canals. The directionality of Exu particle transport through the ring canals thus depends on the position of the ring canal within the egg chamber. These observations suggest that two previously uncharacterized populations of microtubules mediates exu transport, and that the net polarity of these microtubules depends on the position of the ring canal along the anterior-posterior axis of the egg chamber.

**PROFILIN IS REQUIRED TO REPRESS
MICROTUBULE-BASED CYTOPLASMIC STREAMING
WITHIN THE DROSOPHILA OOCYTE.** Lynn Manseau
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The actin and tubulin cytoskeletons both play important roles in *Drosophila* oogenesis. In the final stages of oogenesis, the actin cytoskeleton mediates dumping of the nurse cell contents into the oocyte cytoplasm, which is then mixed by microtubule-based cytoplasmic streaming within the oocyte. *cappuccino* (*capu*) and *spire* (*spir*) are genes required for localization of molecular determinants within the developing *Drosophila* oocyte. *capu* and *spir* mutant oocytes have abnormal microtubule distributions and premature microtubule-based cytoplasmic streaming within the oocyte. Neither the speed nor the timing of this streaming appears to correlate with the strength of the mutant allele, making it unclear whether the streaming is the cause of the mislocalization of determinants in *capu* and *spir*.

Both the abnormal microtubule distribution and premature cytoplasmic streaming phenotypes of *capu* and *spir* can be mimicked by bathing egg chambers in cytochalasin D, a microfilament inhibitor. Because cytochalasin D can induce premature cytoplasmic streaming, we have examined mutants known to affect the actin cytoskeleton during oogenesis for premature cytoplasmic streaming within the oocyte. We have found that mutants in profilin, encoded by the gene *chickadee*, also exhibit premature, microtubule-based cytoplasmic streaming within the oocyte. Mutants in villin, encoded by *quail*, in fascin, encoded by *singed*, and in plakoglobin, encoded by *armadillo* do not exhibit this phenotype.

GENETIC ANALYSIS OF ACTIN DYNAMICS DURING *DROSOPHILA* OOGENESIS

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Throughout most of *Drosophila* oogenesis, nurse cell cytoplasm flows slowly into the oocyte while near the end of oogenesis nurse cells contract and rapidly inject their remaining cytoplasm into the oocyte. Cytoplasm, including organelles, passes through intercellular bridges called ring canals to reach the oocyte. The nurse cell nuclei are excluded from the oocyte during the final regression of the nurse cells. This is accomplished by the presence of extensive networks of cytoplasmic actin filament bundles that form in nurse cells just before final nurse cell regression. These bundles are anchored in the nurse cell plasma membranes and nuclear membranes and provide structural support to hold the nuclei within the cells as they contract. We are studying several genes that are required for formation of the cytoplasmic actin bundles. Mutations in these genes disrupt bundle formation and this allows the nurse cell nuclei to block the ring canals and stop the flow of cytoplasm. The mutants are therefore female sterile because full-sized oocytes are not formed.

Two of the genes in this class, *quail* and *singed*, encode proteins that bundle actin filaments. The *singed* protein is homologous to sea urchin fascin. To analyze functional domains of *singed*, we have made 15 new alleles of *singed* by EMS mutagenesis. We are currently sequencing the alleles and will test corresponding point mutations *in vitro* for actin bundling. We are also taking a genetic approach to understanding *singed* function. We are using an allele of *singed* that produces non-functional protein in a screen for suppressor mutations in other genes. This screen should identify proteins that interact with *singed*.

The *quail* protein has extensive sequence similarity to vertebrate villin. It co-localizes with nurse cell actin bundles *in vivo* and quail protein made in *E. coli* can bundle actin filaments *in vitro*. However, the bacterially-produced protein does not sever actin filaments in a calcium sensitive manner. We can rescue the *quail* mutant phenotype by germline transformation of a *quail* cDNA construct. To test the requirement for actin bundling *in vivo*, we are making a transgene lacking the headpiece region of quail ("headless") and testing its ability to rescue *quail* mutants. To further define functional domains of quail, we are sequencing *quail* EMS alleles. We have begun a second-site suppressor screen, similar to the one for *singed*, using an allele of *quail* with a point mutation.

THE DROSOPHILA 95F UNCONVENTIONAL MYOSIN IS A CYTOPLASMIC MOTOR THAT IS REQUIRED FOR ORGANIZATION OF THE SYNCYTIAL BLASTODERM EMBRYO. Valerie Mermall and Kathryn G. Miller. Department of Biology, Washington University, St. Louis, MO 63130

As part of an effort to understand the actin-mediated, dynamic processes involved in embryogenesis, we previously characterized the gene encoding a new myosin, *Drosophila* 95F myosin heavy chain (95F MHC). In the early embryo, 95F MHC is enriched in particulate structures that rearrange in cell-cycle-coordinated manner. During interphase the particles are present throughout the cytoplasmic domain that surrounds each syncytial nucleus, while during mitosis, they associate with transient membrane invaginations (mitotic furrows) that divide neighboring mitotic apparatus. We have shown that this myosin catalyzes transport of the particles with which it associates in the syncytial embryo.

When 95F myosin function is inhibited by antibody injection, profound defects in syncytial blastoderm organization occur. This disorganization is seen as aberrant nuclear morphology and position, and is suggestive of failures in cytoskeletal function. Nuclear defects correlate with gross defects in the actin cytoskeleton including indistinct actin caps and furrows, missing actin structures, abnormal spacing of caps and small mesh furrows. Examination of anti-95F myosin injected embryos in three-dimensions reveals that actin furrows do not invaginate as deeply into the embryo as do normal furrows. These furrows do not separate adjacent spindles since microtubules cross over them. These inappropriate microtubule interactions lead to aberrant nuclear divisions and to the nuclear defects observed. We propose that 95F myosin function is required to generate normal actin-based transient membrane furrows. The motor activity of 95F myosin itself and/or components within the particles transported to the furrows by 95F myosin may be required for normal furrows formation.

THE ORIENTATION AND FORMATION OF AXES OF CYTOSKELETAL POLARITY John Chant, Lisa Stowers, Michelle Mischke, and Toby Freedman. MCB, Harvard University, Cambridge, MA, USA.

We are interested in how cells polarize their cytoskeletons as controlled by internal programs or external signals. In yeast, two classes of proteins have been defined which are involved in controlling cell polarity. The first class (typified by the BUD proteins) is involved in guiding the orientation of axes of polarity, but is dispensable for formation of axes. The second class (including CDC24, CDC42, CDC43 and BEM1) is required for the formation of axes of polarity. Here, we present progress in understanding of how axes of polarity are oriented by the BUD proteins of yeast and how the human version of ras-related GTPase CDC42 (important for establishing polarity in yeast) can govern the polarization of mammalian T cells towards antigen-presenting cells.

Yeast cells divide by budding in two patterns of high fidelity: an axial pattern (a and α cells), in which cells form buds at the mother-daughter junction of the previous division, and a bipolar pattern (a/α cells), in which cells bud at the poles of their ellipsoidal shapes. We have found that, in the axial pattern, BUD3 assembles in a double ring encircling the mother-bud neck around the time of onset of mitosis. BUD3 probably assembles on the 10-nm filaments, a cytoskeletal structure which encircles the neck. The assembly of BUD3 in the neck region produces a spatial memory in one cell cycle to guide polarization of the cytoskeleton, bud formation and cell division in an axial orientation in the next cell cycle. How the poles of the cell are defined for bipolar budding is currently under investigation.

CDC42 is a ras-related GTPase required for establishing an axis of polarity in yeast. A human version of this protein is known which is >80% identical to the yeast protein. By expression of dominant interfering and activating alleles of *CDC42* in mammalian cells, we have shown that CDC42 is important for orienting both the actin and microtubule cytoskeletons of T cells towards antigen-presenting cells, an external cue. Thus, it appears that some of the mechanisms governing the polarization of the cytoskeleton are conserved in detail between yeast and mammalian cells.

A MUSCLE-SPECIFIC VARIANT OF MICROTUBULE-ASSOCIATED PROTEIN 4 (MAP 4) IS EXPRESSED EARLY IN MYOGENESIS

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Microtubule-associated protein 4 (MAP 4) transcripts vary in different mouse tissues, with striated muscle (skeletal and cardiac) preferentially expressing 8 and 9 kilobase transcripts relative to the more common 5.5 and 6.5 kilobase transcripts (West et al., J. Biol. Chem. 266:21886. 1991). Cloning of the sequence unique to the muscle transcripts demonstrated these mRNAs vary from the more ubiquitous ones by a single 3.2 kilobase coding region insertion within the projection domain of MAP 4. This sequence is expressed only in skeletal and cardiac tissues in adult mouse. Using the myogenic cell line C₂C₁₂, northern analysis showed that the muscle variant sequence appears within ~12-16 hours of growth in differentiation medium, but lags behind myogenin expression by 4-8 hours. During differentiation, there is a concomitant decrease in the expression of the more ubiquitous 5.5 and 6.5 kilobase MAP 4 transcripts. Fusion-inhibited cultures did not express the muscle transcripts. Antisense technologies were used to affect expression of the muscle-specific transcripts in myogenic cultures. A stable C₂C₁₂ cell line bearing a plasmid containing flipped muscle-specific sequence, cells injected with the flipped construct, and cultures treated with antisense oligonucleotides were examined. In all cases, myoblasts were unaffected, but differentiating myotubes showed aberrant morphology. Phenotypes included multinucleate cells that were not polarized, contained large vacuoles, and had bundles of microtubules surrounding the nuclei. In situ hybridization analyses of mouse embryos showed that muscle MAP 4 transcripts were first detectable at embryonic day 11 in pre-thoracic muscle and caudal somites, and that expression continued to expand into and persist in developing skeletal muscle structures through day 16. The antisense experiments and the patterns of expression of these muscle-specific transcripts both in vitro and in vivo suggest that this unique form of MAP 4 plays a critical role in early myogenic events, and is also required in adult tissue. (Supported by NIH GM22214 to JBO)

CYTOSKELETAL DYNAMICS AND BIOGENESIS OF CYTOARCHITECTURE IN CARDIOMYOCYTES.

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In freshly explanted adult rat cardiomyocytes (ARC) the sarcomeric organization breaks down, but the cells survive and later the regeneration of sarcomers is dependent on intact microtubular and microfilament networks. In their absence, the myofibrillar components are degraded, while protein synthesis is normal. Neonatal cells behave differently and have more stable myofibrils. During cardiogenesis, several proteins are newly expressed that have no counterparts in non-sarcomeric cells, as for example the M-band component myomesin which may play a pivotal role in myofibrillogenesis. cDNA clones were obtained and this protein was further characterized.

Most contractile proteins form complex isoprotein families, as e.g. the actins, myosin heavy chains, myosin light chains, and are expressed in temporal sequences ranging from the nonmuscle isoform in undifferentiated cells to various sarcomeric isoforms in differentiated cardiomyocytes. The functional differences of each of these isoproteins is not well understood. The actin and the MLC isoforms were investigated more thoroughly. While the sarcomeric actin filaments in regenerated ARC are stable, the non-sarcomeric (cytoskeletal) actin filaments are disorganized by the treatment with Cytochalasin D. Furthermore, the additional expression of muscle actins does not influence the ARC cytoarchitecture significantly. The sarcomeric actin thin filaments however, can be influenced by the microinjection of cDNA encoding either β - or γ -cytoplasmic actin. A dominant isoprotein specific effect can be observed leading to destabilization of thin filaments, while the organization of thick filaments and thick filament associated components is not significantly altered.

During many developmental processes, isoforms are changing with maturation of cellular organisation. The relative specificity of binding of various MLC isoproteins to myofibrils was determined, using transfection of pairs of MLC isoprotein cDNAs labeled with different epitope tags. The expression and intracellular distribution of the proteins within single cardiomyocytes were analyzed by double immunofluorescence. While the non-muscle alkali MLC has the lowest specificity for sarcomeric interaction, the fast muscle isoforms were bound with higher specificity than even MLC 1sb, the isoform which is expressed endogenously. This intra-compartmental sorting specificity may in part explain one function of members of this isoprotein family: their differential specificity for interaction with cell specific structures. It is likely that such hierarchies exist for many other contractile isoprotein families facilitating exchange during development and turnover, leading to modification of organelle functions during biogenesis of cardiac cytoarchitecture.

BETA BARRELS, MYOSIN BINDING PROTEINS AND SARCOMERE ASSEMBLY ^{*}Fischman, D.A., ^{*}Gilbert, R., ^{*}Alyonycheva, T., ^{*}Lebedeva, M., ^{*}Mikawa, T., ^{*}Whittle, M.R. and ^{**}Reinach, F.C., ^{*}Department of Cell Biology and Anatomy, Cornell University Medical College and [#]Department of Biochemistry, University of Sao Paulo.

The myosin binding domains of three proteins: skeletal MyBP-C, skeletal MyBP-H and cardiac MyBP-C reside within single IgC2 motifs comprising the C-terminal, ~100 amino acids of each protein. It is likely that all of these domains are organized in β -barrel configurations composed of anti-parallel β -sheets, analogous to those crystallized in the CAMs, lymphocyte receptors and cadherins. These regions of the MyBPs also underlie the specificities of binding to different myosin II isoforms, i.e., the C-terminal 14kD fragments of skeletal MyBP-C and skeletal MyBP-H bind with higher limiting stoichiometries to skeletal than cardiac myosin. The converse is true for cardiac MyBP-C: this protein binds in greater amounts to cardiac than skeletal myosin. Using a panel of MyBP-C truncation mutants and transient transfections of embryonic chick myoblasts, we have shown that the C-terminal region of ~350 amino acids of MyBP-C contains all of the information both necessary and sufficient for the targeting of this protein to the A band of the sarcomere. In myotubes expressing a truncation mutant lacking the last IgC2 motif there appears to be an inhibition of myofibril formation; i.e., this construct may be a dominant-negative mutant of sarcomere assembly. Since MyBP-C is a late appearing protein during myofibrillogenesis, it could be rate limiting in myofibril assembly, possibly functioning in the lateral alignment of thick myofilaments in nascent A bands. The major site of interaction between MyBP-C and myosin is within the amino terminal one-third of LMM: deletion of this region of the myosin rod has a dramatic inhibition on its binding to MyBP-C. The human gene encoding skeletal MyBP-H has been fully sequenced and its exon and intron boundaries mapped. Each of the IgC2 and Fibronectin III motifs of the protein are encoded by two exons, each of which encodes for one-half of each β -barrel. A mouse gene syntenic with the human gene for MyBP-H has been isolated and partially sequenced. A construct has been made to permit homologous recombination and gene knockout in mice. [Supported by grants from NIH, MDA, AHA and the Mathers Foundation.]

TEST OF THE IMPORTANCE OF A HYDROPHOBIC PLUG IN THE STABILIZATION OF THE F-ACTIN HELIX

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The Holmes model of the F-actin helix predicts that a hydrophobic plug (residues 265-268) at the end of a loop between subdomains 3 and 4 inserts into a hydrophobic pocket at the interface of two actin monomers on the opposing strand resulting in cross-strand stabilization of the actin helix. To test this theory, we have used site-directed mutagenesis to make a series of mutations in this plug in yeast actin to alter its hydrophobicity and have assessed the effects of these mutations *in vivo* and *in vitro*. This plug in yeast actin is V₂₆₅-L-G-L, and the nature of the pocket predicts that V₂₆₅ should be in the most hydrophobic environment and L₂₆₈ in the least. Diploid cells with one disrupted and one normal actin gene were transformed with the mutant actin cDNA on a centromeric plasmid. Transformed cells were allowed to sporulate and surviving haploid cells expressing only the mutant actin were isolated for further study. Of the diploid cells, those with the V₂₆₅F, V₂₆₅G, L₂₆₈G and V₂₆₅G/L₂₆₈G (GG) mutations recovered from transformation as fast as those with wild-type actins and much faster than one with the V₂₆₅D mutation. Of these, only the VD cells were nonviable as haploids, and the VD/WT diploid cells were osmosensitive. VG cells showed no temperature sensitivity but displayed an altered budding pattern. Of the haploid cells, only the GG cells were osmosensitive, and they were very cold sensitive at 10° (71 hr. vs. 25 hr. doubling time). Previously, we observed that polymerization of the L₂₆₈D mutant actin *in vitro* was markedly cold sensitive compared with wild-type actin. VG and LG actins did not display this property. However, GG actin at 0.8 mg/ml did not polymerize at temperatures < and only marginally at 25°. Addition of wild-type F-actin seeds did not promote polymerization. However, a mixture of 0.4mg/ml each of wild-type and GG actin polymerized at 25° to nearly the same extent as 0.8 mg/ml wild-type actin. These results are consistent with the predictions of Holmes and suggest that an energy threshold must be crossed before weakening of the plug-pocket interaction is sufficient to cause substantial helix disruption.

**STRUCTURE/FUNCTION STUDIES OF ACTIN AND
ACTIN BINDING PROTEINS, D.C. Amberg and D. Botstein,**
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We have developed an *in vivo* system for characterizing how actin binding proteins interact with yeast actin. This system tests for the ability of actin binding proteins to bind to a synoptic set of actin mutants in the protein interaction reporter system known as the two-hybrid system. In this manner, we have characterized the actin interactions of several known or suspected actin ligands including: actin itself, Srv2 (a.k.a. cyclase associated protein), profilin, Sac6 (yeast fimbrin), Rvs167 and Fus1 (both SH3 domain containing proteins). In addition, we have discovered that the flavin containing oxidoreductase called "old yellow enzyme" and 3 previously unidentified proteins (AIP1, 2 and 3) interact with actin in the two-hybrid system.

Those mutations which disrupt a given actin:actin binding protein interaction can be thought of as providing a fingerprint that characterizes the interaction thus allowing us to compare how different ligands bind actin. Using this criterion we found that most of the ligands we are studying appeared to interact with actin in different manners. However, we found that the profilin and Rvs167 data was nearly identical suggesting a common method of interaction with actin.

In some cases, projection of alleles that disrupt ligand binding on the structure of actin, delineated regions of interaction for actin binding proteins. Modeling of the mutations that disrupt the actin:actin interaction suggested we were detecting a back to back dimer of actin analogous to the interaction proposed to occur between strands of the actin filament in the Heidelberg model. The data for the Sac6-actin complex suggested Sac6 was binding on subdomains 1 and 2 of an actin dimer, consistent with data from other methods. The data for the Aip1 interaction suggested a binding site on subdomains 3 and 4.

The information obtained in our analysis is different than the data obtained from more classical methods used to study multi-protein complexes such as X-ray crystallography. However, the speed and accessibility of this methodology to researchers of widely different backgrounds makes our system a useful addition to the tools used to study interactions with the actin cytoskeleton.

EFFECTS ON ACTIN ASSEMBLY AND CELL MOTILITY IN DICTYOSTELIUM CAPPING PROTEIN MUTANTS. Christopher Hug^{*}, Patrick Y. Jay[‡], Indira Reddy^Δ, James G. McNally^Δ, Paul C. Bridgman[§], Elliot L. Elson[‡], and John A. Cooper^{*}. Departments of Cell Biology and Physiology^{*}, Biochemistry and Molecular Biophysics[‡], Anatomy and Neurobiology[§], and Biology^Δ, Washington University, St. Louis, MO

Actin assembly is important for cell motility, but the mechanism of assembly and how it relates to motility in vivo is largely unknown. In vitro, actin assembly can be controlled by proteins, such as capping protein, that bind filament ends. To investigate the function of actin assembly in vivo, we altered the levels of capping protein in Dictyostelium cells, and found changes in resting and chemoattractant-induced actin assembly that were consistent with capping protein's in vitro properties of capping but not nucleation. Significantly, over-expressers moved faster and under-expressers moved slower than control cells. Mutants also exhibited changes in cytoskeleton architecture, mechanical properties, phagocytosis, growth rate and ploidy. These results provide new insights into in vivo actin assembly and the actin cytoskeleton's role in motility.

ACTIN-BASED MOVEMENT OF LISTERIA MONOCYTOGENES IN XENOPUS EGG EXTRACTS IS DUE TO LOCAL UNCAPPING OF THE BARBED ENDS OF ACTIN FILAMENTS AT THE BACTERIUM SURFACE AND RESULTING SHIFT IN STEADY-STATE OF ACTIN ASSEMBLY.

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The thermodynamic basis for actin-based motility of *Listeria monocytogenes* has been investigated using cytoplasmic extracts of *Xenopus* eggs, initially developed by Theriot *et al.* (1994, Cell 76, 505-517) as an *in vitro* cell-free system. A large proportion (75 %) of actin was found non polymerized in the extracts. The amount of unassembled actin (12 μ M) is accounted for by the sequestering functions of TB_4^{Xen} (20 μ M) and profilin (5 μ M), the barbed ends being capped. Movement of *Listeria* is unaffected by depletion of over 99 % of the endogenous profilin. The pro-rich sequences of ActA are unlikely to be the target of profilin. All data support the view that actin assembly at the rear of *Listeria* results from a local shift in steady state due to a barbed end uncapping activity bound to the surface of the bacterium, while barbed ends are capped in the bulk cytoplasm. Movement is controlled by the energetic difference (i.e. the difference in critical concentration) between the two ends, hence a constant ATP supply and the presence of a minimum amount of F-actin in the medium are needed to support the movement.

The role of membrane components is demonstrated by the facts that 1) *Listeria* movement can be reconstituted in the resuspended pellets of high speed-centrifuged extracts; 2) Actin-based motility of endogenous vesicles, exhibiting the same rocketing movement as *Listeria*, can be observed in the extracts; 3) Movement of *Listeria* can be arrested by pretreatment of the extracts by phosphatidylinositol-specific phospholipase C from *B. cereus*.

THE USE OF EPITOPE TAGGING TO STUDY TROPOMYOSIN ISOFORM DIVERSITY: ALTERNATIVELY SPliced EXONS FUNCTION IN PART TO REGULATE HOMODIMER VERSUS HETERODIMER FORMATION

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Tropomyosins (TMs) are a family of F-actin binding proteins with distinct isoforms expressed in specific cell types. For example, skeletal and smooth muscle cells express two isoforms of TM while fibroblasts express 6-8 distinct isoforms. In vertebrates four genes are known to encode at least 16 distinct isoforms, in large part via a complex pattern of tissue-specific alternative RNA splicing. In order to further study the function of this diversity, cDNA clones to the different isoforms were cloned into eukaryotic expression vectors containing either the 23 residue HA-tag or the 11 residue VSV-G-tag at their N-termini. The immunofluorescent staining patterns of all transfected isoforms were indistinguishable from those obtained for endogenous TM, indicating the integration into actin filaments. The tagged TMs expressed in *E. coli* co-sedimented with actin in *in vitro* assays demonstrating that the tag did not interfere with the ability to bind to F-actin.

TM is a dimer composed of two coiled-coil polypeptide chains with close to 100% α -helical content. Previous studies have shown that TMs from fibroblasts form homodimers, while TMs from skeletal muscle or smooth muscle preferentially form heterodimers. To investigate the coiled-coil interaction of TMs and to determine if cell type-specific factors are responsible for the control of homo- or heterodimerization *in vivo*, we have singly or doubly transfected various cell lines to study the association of TMs. Following transfection, the tagged TMs were immunoprecipitated from a total cell extract using a monoclonal antibody against the HA sequence. Our results demonstrate, that both high (HMW) and low molecular weight (LMW) fibroblast TMs form exclusively homodimers as indicated by the presence of both tagged and untagged molecules of a given TM isoform in the precipitate. Simultaneous co-transfection of muscle isoforms (α -smooth or β -skeletal) into fibroblasts together with nonmuscle isoforms TM-1, TM-2 or TM-3 however, resulted in the precipitation of both nonmuscle and muscle TMs, demonstrating the ability of the latter to form heterodimers with nonmuscle isoforms. Co-transfection of either muscle or nonmuscle HMW (284 amino acids) isoforms with LMW isoforms (248 amino acids) TM-4, TM-5a or TM-5b, in contrast, showed no interaction between TMs of different chain length. Our data indicate that the alternatively spliced exons function, in part, to regulate the dimeric state of TM. The implications of these results to cellular function will be discussed. Furthermore, these studies demonstrate the usefulness of epitope tagged TMs to study the properties of the individual isoforms.

THE TROPOMODULIN INDUCED INCREASE IN THE POINTED END
CRITICAL CONCENTRATION REQUIRES ENERGY INPUT FROM ATP
HYDROLYSIS.

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We showed recently that tropomodulin caps the pointed ends of actin filaments (Weber et al. 1994, J. Cell Biol. 127:1627-1635). In the presence of tropomyosin, tropomodulin in very low concentrations completely blocks elongation and depolymerization from the pointed filament end. In the absence of tropomyosin, when tropomodulin can only bind to actin, it forms a "leaky" cap. That is, in saturating concentrations it lowers the rate of elongation to about 1/5th of the control rate and it reduces the apparent rate constant of depolymerization by about a factor of 2. Tropomodulin also increases the critical concentration of gelsolin-capped actin filaments. This is quite different from the effect of barbed-end-capping proteins on the critical concentration which is due to a shift of the critical concentration from that of the barbed end to that of the pointed end when the barbed ends are completely capped. By contrast, tropomodulin increases the apparent K_d of the pointed end for actin. Thermodynamic considerations suggest, although this is not intuitively obvious, that this effect of tropomodulin requires energy input, in this case the hydrolysis of ATP, the only available energy source in these assays.

This idea is supported by our finding that tropomodulin does not raise the critical concentration of gelsolin-capped actin filaments when the assay is free of ATP and all of the actin has been converted to ADP-actin. However, tropomodulin still inhibits elongation and depolymerization of ADP-actin, demonstrating that tropomodulin's pointed end capping activity itself does not require ATP. In the presence of ATP, the actin molecules at the filament end are converted from actin-ATP to actin-ADP·Pi right after incorporation into the filament, and afterwards much more slowly to actin-ADP. Since actin-ATP has been shown to be very short-lived we checked whether the conversion of actin-ADP·Pi to actin-ADP was essential for the tropomodulin effect on the critical concentration. In order to prevent the progression from actin-ADP·Pi to actin-ADP we added 50 mM inorganic phosphate to ATP in the medium (pH 7.0). This abolished the tropomodulin effect on the critical concentration although tropomodulin continued to act as a leaky cap at the pointed filament end.

We tentatively conclude that the input of energy required for the tropomodulin effect on the critical concentration at the pointed filament end is derived from the release of inorganic phosphate from ADP·P actin at the filament end. More data are needed to understand the mechanism by which this energy input increases the critical concentration and the mechanism responsible for the leakiness of the cap.

LOCALIZATION OF THE THIRD ACTIN BINDING SITE IN GELSOLIN AND IDENTIFICATION OF CALCIUM SITES.

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Gelsolin contains six repeating segments of sequence (G1-6) based on their pattern of conserved residues. Although the intact protein binds two actin monomers, three distinct binding sites have been identified using proteolytic fragments or bacterially expressed domains. Segment 1 (G1) contains the high affinity calcium-independent actin monomer binding site while the filament binding domain is in G2. These two domains (G1-2) form the minimal severing unit, which also caps the barbed ends of actin filaments (Way *et al.*, *J. Cell Biol.* 119, 835-842 (1992)). The third binding site, the calcium dependent G-actin site is in G4-6, but its exact location has not been identified. This site is required for the nucleating activity of gelsolin. Here we have localised the third site to G4, the segment showing the highest conservation of amino acid sequence compared to G1. Constructs containing G4, G5, G4-5 and G5-6 were expressed in *E. coli*. Both G4-5 and G4 bind G-actin, but there is no binding by G5 or G5-6.

Gelsolin binds 2 calcium ions with $K_d \sim 1 \mu M$ (Weeds *et al.*, *Eur. J. Biochem.* 161, 69-76 (1986)), but these sites have not been localised to specific segments. Using equilibrium dialysis, 2 calcium sites have been identified in G4-6, one of which binds calcium about ten times more strongly than the other. Analysis of calcium binding by the segments above showed that G5-6 contains the high affinity binding site while G4-5 binds calcium with lower affinity. Neither G4 nor G5 on their own bound calcium. Thus the calcium switch that activates gelsolin is located in G6 or G5-6. Binding of calcium at this site makes G4 accessible to actin and this in turn co-operatively facilitates binding at the other, higher affinity sites in G1 and G2, thereby activating severing or nucleation.

A single calcium ion that cannot be removed by EGTA is "trapped" in the complex between actin and gelsolin (Bryan and Kurth, *J. Biol. Chem.* 259, 7480-7487 (1984)). A single calcium was also found in the G1:actin complex (Way *et al.*, *EMBO J.* 9, 4103-4109 (1990)). By contrast, two calcium ions were located in the structure of this complex solved by X-ray analysis at 2.5 Å resolution (McLaughlin *et al.*, *Nature* 364, 685-692 (1993)). One was bound intramolecularly in G1 and the other co-ordinated intermolecularly between G1 and actin. Using site directed mutagenesis, the intramolecular calcium has been identified as the ion bound in the complex.

The implications of these various findings for the functions of gelsolin will be discussed.

HUMAN MACROPHAGE CAP G: CHARACTERIZATION OF GAIN-OF-FUNCTION MUTANTS WITH ACTIN-SEVERING ACTIVITY F. S. Southwick and M. R. Bubb, Departments of Medicine and Biochemistry & Molecular Biology, University of Florida College of Medicine Gainesville, FL 32610-0277

Nonmuscle cell motility requires marked changes in the consistency and shape of the peripheral cytoplasm. These changes are regulated by a gel-sol transformation of the actin filament network, and actin filament-severing proteins are responsible for network solation. Macrophage Cap G, unlike all other proteins in the gelsolin family, caps but does not sever actin filaments. We took advantage of Cap G's inability to sever filaments to identify amino-acid sequences likely to play important roles in actin filament severing. We reasoned that any amino-acid sequence shared by the four severing proteins, gelsolin, villin, severin and fragmin, but not by Cap G, should identify sites most likely to be required for severing activity. Two amino acid stretches in Cap G diverge markedly from the severing proteins: 84-LNTLLGE and 124-AFHKTS. Discrete mutations in Cap G have been generated by PCR to determine if these amino-acid sequences are critical for actin filament severing. The mutant and wild type proteins have been expressed in the pET12a vector and purified by FPLC ion exchange chromatography. Conversion of 84-LNTLLGE to the gelsolin actin-binding-helix sequence (84-LDDYLGG) renders Cap G capable of severing actin filaments (half maximal severing at 1-2 μM , as measured by depolymerization of gelsolin capped pyrenyl F-actin). While conversion of 124-AFHKTS to 124-GFKHV does not result in severing (no severing up to 3 μM), addition of this mutation to the LDDYLGG severing mutant did enhance severing by 10 fold (half maximal severing 0.1-0.2 μM). Enhancement of severing function could be the result of increased actin filament side-binding activity. Actin filament pelleting assays, however, demonstrate that the GFKHV mutation does not detectably increase actin filament side-binding activity as compared to wild type protein. The barbed end filament capping affinity of each mutant has also been examined. The K_D for capping (0.5 nM) was identical to wild type protein for all the mutant proteins, indicating that different structural determinants mediate the capping and severing of actin filaments. We are presently using pyrenyl-actin in fluorescence and velocity sedimentation experiments to characterize the actin monomer binding properties of mutant and wild-type CapG. Future studies of these gain-of-function mutants promise to provide further insight into how phagocytic cells sever actin filaments to generate the shape changes critical for amoeboid movement.

THE EFFECT OF F-ACTIN ON THE PROTEIN SYNTHETIC ACTIVITY OF ELONGATION FACTOR-1A.

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Many studies implicate the actin cytoskeleton in mRNA translation. Consequently it is intriguing that ABP50, a major actin binding/bundling protein from *Dictyostelium*, is the eukaryotic elongation factor-1 α (EF1 α) with 75% identity to other EF1 α s. Evidence that EF1 α s from a variety of species display identical actin binding activity demonstrates that actin binding is a highly conserved property of all EF1 α s and suggests that actin is important for EF1 α function. These observations raise the issue of the potential regulation of protein synthesis by the cytoskeleton. The current model of eukaryotic translation presents EF1 α binding aminoacyl-tRNA to the ribosome by a GTP-regulated mechanism. The proposed steps involving EF1 α are: (i) guanine nucleotide exchange, (ii) complex formation with aminoacyl-tRNA, and (iii) EF1 α -tRNA complex binding to the ribosome. We have found that F-actin crosslinked by EF1 α preferentially affects (i) with little effect on (ii) or (iii); F-actin stimulates the exchange by EF1 α of bound GDP for free GTP. This result demonstrates the potential of F-actin to facilitate polypeptide elongation by enhancing the recycling of EF1 α to its active GTP-bound state. Further observations that tRNA and ribosomes reduce the binding of F-actin by EF1 α suggest that ribonucleic acid and F-actin binding are mutually exclusive events in agreement with the proposed actin binding sites of EF1 α . Our working model indicates that polypeptide elongation during the EF1 α cycle doesn't require a constant association with F-actin although a transient association is comensalistic.

**Isolation and characterization of a peptide that disrupts
the talin-vinculin interaction**

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Focal contact associated cytoskeletal proteins were immobilized on plastic and screened using phage-displayed random peptide libraries. These experiments produced phage that bound to the cytoskeletal protein vinculin. One phage-displayed peptide, pV12-1, was chemically synthesized and tested for binding to fragments of vinculin expressed in *E. coli*. The results showed that the peptide interacts with vinculin fragments containing the amino-terminal 252 amino acids; this region has been previously shown to contain a talin binding domain. In competition experiments, pV12-1 blocked the interaction of immobilized vinculin with talin but not with paxillin or α -actinin, two other vinculin-binding proteins. Solution binding assays indicated the peptide will only bind to fragments of vinculin lacking the carboxy-terminus. This result is consistent with previous findings that show an intramolecular interaction between the amino-terminus and carboxy-terminus of vinculin masks the talin-binding domain and suggests immobilization of vinculin can unmask this domain. pV12-1 shares three sequence motifs with a region of talin that contains a vinculin-binding domain. Saturation mutagenesis of pV12-1 displayed on phage indicates mutations in these motifs disrupt binding. We are currently employing site-directed mutagenesis to determine if changes in the motifs found in talin will also disrupt binding. In summary, this study produced a peptide that interacts with a talin-binding domain of vinculin, and illustrates how random peptide libraries can be a source of reagents for the study of protein-protein interactions.

THE SIGNIFICANCE OF THE MICROTUBULAR SYSTEM IN THE RESPONSE OF EPITHELIAL CELLS TO HGF/SF.

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Scattering of the MDCK epithelial cells induced by scatter factor (HGF/SF) provides a good system for investigation of spreading, polarization and directional motility of cells. The aim of our experiments was to examine the role of microtubular system in HGF/SF induced reorganization. We used two microtubule-specific drugs - taxol and colcemid - which destroyed the microtubular system of cells by the different way. To estimate the morphological changes after HGF/SF treatment with (or without) taxol and colcemid we used the elongation and dispersion indices proposed by Dunn and Brown (1986, J. Cell Sci., 83: 313). We observed the reorganization of cytoskeleton after different treatment using indirect immunofluorescence microscopy. To study the migration properties of the cells with intact and destroyed microtubular system induced by HGF/SF we used Boyden chambers. The results suggest two stages in the response to HGF/SF: a) activation of the extension of lamellae leading to cell spreading and b) reorganization of microtubules, leading to polarization of cell shape. These two components have different sensitivities to microtubule-specific drugs, especially to taxol. Treatment of the cells with these drugs simultaneously with HGF/SF did not prevent the increase of cell area and lamella formation during the first stage, but inhibited the increase of dispersion and elongation (and so the degree of polarization) during the second stage. Taxol also inhibited the dispersion of the circular bundle of microfilaments after HGF/SF treatment. However taxol-induced block of polarization did not inhibit activation of cell movement through the filters. Obviously, general activation of extension and substratum attachment of the lamellae induced by HGF/SF without polarization was sufficient to stimulate cell migration. We are deeply grateful to Prof. J. Vasiliev and Dr. E. Gherardy for general help and useful discussions. We are also grateful to Mrs. K. Lane for kind help in our experiments.

ACTIN FILAMENT ATPASE ACTIVITY INFLUENCES SEVERING BY GELSOLIN.

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Hydrolysis of ATP by actin is stimulated by monomer addition to the filament, yet this hydrolysis is not necessary for filament polymerization. Nucleotide hydrolysis may function to regulate association of actin binding proteins. While ATP hydrolysis is irreversible, it is possible to regenerate the intermediate state of ADP-Pi bound to the filament. Gelsolin and its genetic siblings are members of one class of actin filament proteins which can non-proteolytically sever F-actin. We find that the generation of the hydrolysis intermediate ADP-Pi F-actin by addition of phosphate, AIF or BeF to ADP-F-actin reduces the ability of gelsolin to sever the filament. While a binding site for H_2PO_4^- has been described on F-ADP actin, the pH sensitivity of this effect would argue for the binding of HPO_4^{2-} .

Previous studies have suggested the presence of two phosphate binding sites. Similarly, the fluorescence change of F-ADP pyrene actin exposed to various concentrations of BeF also suggests the presence of two binding sites for this molecule. These data suggest that: there are two potential phosphate or phosphate analog binding sites on F-ADP actin. This implies that a freshly polymerized actin filament, where one of these sites would be filled with Pi, would be resistant to severing by gelsolin (and, from previously published data, cofilin-like molecules). Ongoing work is attempting to test this hypothesis directly. (Supported by a Fellowship from the American Heart Association, Massachusetts affiliate).

ANNEXIN I - PROFILIN INTERACTIONS: INVOLVEMENT IN MEMBRANE-CYTOSKELETON REORGANIZATION ?

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Interactions between the cytoplasmic membrane and the cytoskeleton are known to play important roles in cellular functions. Annexins constitute a family of proteins which bind phospholipids and membrane in a calcium dependent manner. Annexin I which is thought to be involved in processes implicating membrane fusion such as phagocytosis may therefore participate in membrane-cytoskeleton reorganization. In looking for annexin I binding proteins, we found that this protein can interact with profilin, an actin binding protein known to play important functions in the modulation of actin filaments as well as in transmembrane signaling. 1) Interactions of annexin I with profilin are specific and of high affinity as judged by Overlay technique and ELISA method and the resulting complex modifies both annexin I and profilin functions. 2) The binding of annexin I on phosphatidyl-serine containing liposomes is prevented by profilin. 3) This effect is annulled when PIP2 to which profilin is more tightly bound, is included in the liposomes. 4) Finally, annexin I which we found enhances actin filament formation probably in association with an other protein, counteracts the inhibiting activity of profilin on actin polymerization. The modulating activity of calcium in membrane-annexin I-profilin association as well as the possible implication of annexin I - profilin interactions in membrane-cytoskeleton reorganization arising from e.g. binding of an extracellular factor will be discussed.

RELATIONSHIP OF MOESIN AND ACTIN FILAMENTS DURING
PROTRUSION AND RETRACTION OF CELLS IN CULTURE.

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Moesin is localized in filopodia, retraction fibers and other cellular protrusions, where it could provide links between the plasmamembrane and actin filaments. It binds to F-actin in vitro at a site located within its C-terminal sequence. We have now studied in greater detail the relationship between actin filaments and moesin in several cell lines. Time-lapse DIC video recordings were used to distinguish between filopodia and retraction fibers at the cell edges, and to observe growth, transformation into pseudopodia, veils, lamellopodia, or their retraction in different areas of the same cell. After fixation, cells were analysed by indirect immunofluorescence with antibodies to moesin and with rhodamine-phalloidin. Both, moesin and actin filaments are found within filopodia and retraction fibers, but distinct differences in their distributions are of note. Staining intensities of moesin are considerably enhanced in tips of filopodia and in retraction fibers, while more actin signal is apparent at the base of filopodia and in lamellopodia. Interestingly, moesin staining is not observed in newly formed lamellopodia and in (portions of) filopodia that are converting to other forms of protrusions. Experiments with cytochalasin and phorbolesters also support the observed differences in distributions of moesin and actin. We suggest that these localizations of moesin reflect dynamic changes in cytoskeletal organization that occur within filopodia and other active areas of the plasmamembrane, and that this organization varies in different protrusions. Moesin could play a role in the formation, stabilization and maintenance of filopodia by regulating attachment of actin filaments to the plasmamembrane in these structures.

TRACING MECHANOCHEMICALLY ACTIVE PHOSPHOMYOSINS I
DURING *ACANTHAMOEBA* MOTILITY : INSIGHTS INTO MULTIPLE
MYOSIN I ISOFORM FUNCTION .

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The distributions of phosphomyosins IA, IB and IC have been compared with each isoform's total distribution by laser scanning confocal microscopy and quantitative immunogold cytochemistry during phagocytosis and contractile vacuole activity in *Acanthamoeba*. Only phosphorylated myosin I would be expected to be mechanochemically active in the cell (unphosphorylated myosin I has very low actin-activated Mg^{2+} -ATPase *in vitro*). Enhanced chemiluminescence was used to determine total cellular myosin I concentrations of 80 nM for myosin IA, 500 nM for myosin IB and 100 nM for myosin IC. Myosin IA was 70-100% phosphorylated while myosins IB and IC were only 20-30% phosphorylated. Phosphomyosin IA increased by 50%, phosphomyosin IB did not change and phosphomyosin IC decreased by 50% due to phagocytosis. Phosphomyosin IA was concentrated in the cortex around phagocytic cups. Phosphomyosin IB was 5-30-fold concentrated in regions of plasma membrane activity. Since total myosin IB was maximally only 1.5-fold concentrated in these regions, phosphorylation of membrane-associated myosin IB must occur in highly localized regions of the plasma membrane during motility. Myosin IC phosphorylation was coupled to the contractile vacuole cycle and while the total amount of myosin IC associated with the contractile vacuole membrane did not change during the cycle, phosphomyosin IC increased 20-fold during contraction. In general, membrane-associated myosin I was only 10-20% phosphorylated while cytoplasmic myosin I was 60-100% phosphorylated indicating that myosin I in these two compartments may be regulated by different mechanisms. These data, when combined with the previously published differential localization of total myosins I (Baines et al., 1992. JCB 119:1193-1203.), provide insights into the functions of multiple myosin I isoforms in cells.

THE GENERAL FUSION FACTOR TAP/P115/USO1P IS REQUIRED FOR BINDING OF VESICLES TO TARGET MEMBRANES IN EXO- AND ENDOCYTIC MEMBRANE TRAFFIC. M. Barroso and E. Sztul, Dept. Molecular Biology, Princeton University, Princeton, NJ 08544

We have previously described a cell-free fusion assay that reconstitutes the final stage of transcytosis, fusion of transcytotic vesicles with the plasma membrane. We have shown that a 108kD peripheral membrane protein named TAP (Transcytosis Associated Protein), found on transcytotic vesicles, is required for the fusion. Biochemical and electron microscopic analyses indicate that TAP is an extended parallel homo-dimer, similar to myosin II dimers. The TAP dimer contains two globular "heads" (each 9nm in diameter) and an elongated "tail" (45nm in length). Sequence analysis of the open reading frame predicts a 107kD cytosolic protein composed of three structural domains: an N-terminal 72kD globular domain; an internal 32kD dimerization domain containing 4 distinct coiled-coil regions each flanked by helix-breaking prolines and glycines; and a C-terminal 3kD domain containing a preponderance of acidic residues. The predicted domain organization of TAP fits well with the dimensions of the "head" and "tail" domains as defined by EM. TAP is identical to rat p115 and 90% identical to bovine p115. p115 is a recently described cytosolic protein required in conjunction with NSF, SNAPs and other transport factors for *cis* to *medial* Golgi transport of VSV G protein *in vitro*. TAP is also related to Uso1p, a *S. cerevisiae* protein required for endoplasmic reticulum to Golgi transport *in vivo*. The "head-tail-acidic" domain organization of Uso1p and the sizes of the "head" and the acidic regions are similar to those of TAP but the Uso1p "tail" is almost four times the length of TAP's tail. Although the overall homology between TAP and Uso1p is limited, there are three regions of extreme amino acid conservation which appear to represent functional domains of the proteins. We are examining them by mutational analysis. Since TAP is identical to p115 and homologous to Uso1p, we suggest that TAP is a general fusion factor, probably performing an identical function in exocytic and endocytic membrane traffic. This is supported by the distribution of TAP in mammalian cells where TAP is found associated with transcytotic vesicles, secretory vesicles derived from the TGN, within the Golgi complex, and on endosomal compartments. Vesicle targeting and fusion are dependent on ordered reversible interactions between membrane receptors (v-SNAREs and t-SNAREs) and soluble factors (NSF, SNAPs, rabs and various SEC gene products). Where does TAP function? To define the molecular mechanism of TAP action, we modified our *in vitro* transcytotic fusion assay in order to separate the overall reaction into: 1) binding of vesicles to the acceptor membrane and 2) membrane fusion. Using this partial assay we determined that TAP is required for stable binding of vesicles to the target membrane. We propose that TAP might act as a vesicular "anchor" by interacting with the target membrane and holding the vesicular and target membranes in close proximity. Interestingly, other proteins involved in vesicular traffic also contain coiled-coil domains. The transmembrane v-SNARE (VAMP) and t-SNARE (syntaxin and its yeast homolog SED5) and the soluble α - and β -SNAPs contain coiled-coil domains and might form protein-protein interfaces with the coiled-coil regions of TAP. Analyses of such interactions are currently in progress.

A GOLGI-LOCALIZED MEMBRANE SKELETON

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Two functions have been proposed for the spectrin membrane cytoskeleton: the maintenance of membrane structural integrity (the red blood cell) and the formation of discrete cell surface membrane domains (polarized cells). The Golgi apparatus has a requirement for both of these functions. We have identified homologs of erythroid spectrin and ankyrin which localize to the Golgi complex by light and electron microscopy, microinjection of rhodamine-conjugated spectrin, immunoblotting of purified Golgi membranes and expression of cDNA clones. Golgi-localized spectrin and ankyrin are dissociated from Golgi membranes under conditions that perturb Golgi structure and function (mitosis and brefeldin A), indicating a critical role for the Golgi membrane skeleton in Golgi organization. Rapid dissociation following brefeldin A treatment also implies a cycle of protein association and dissociation. Golgi-localized ankyrin is associated with a detergent insoluble structure that is, at the level of the light microscope, morphologically indistinguishable from the Golgi complex. This detergent insoluble "Golgi ghost" is positionally fixed within the cytoplasm through interactions with a colchicine- and cold-stable population of microtubules. Na,K-ATPase, presumably in transit through the Golgi apparatus, is also retained within this Golgi ankyrin ghost. At the ultrastructural level, Golgi ankyrin is associated with small vesicles and tubular structures which reside at one side of the stacked cisternae, possibly representing elements of the trans Golgi network (TGN). This Golgi-localized membrane skeleton could serve to maintain TGN structure; it may also interact selectively with a discrete subset of newly synthesized membrane proteins as they pass through the Golgi complex, thereby acting as a membrane protein sorting machinery.

IMMUNOLOCALIZATION OF KINESIN-LIKE PROTEINS TO THE CONNECTING CILIUM OF FISH PHOTORECEPTORS.

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As part of our studies on flagellar kinesin-like proteins (klps), we have sought to identify klps in the connecting cilium of rod photoreceptors from sunfish retina. The connecting cilium (9 + 0) is the only structural link between the rod inner segment (RIS, the cell body) and the photosensitive rod outer segment (ROS) and is thought to be the channel through which all material passes into and out of the ROS.

We have used two different antibodies (anti-LAGSE and anti-Klp1 head) to the conserved motor domains of klps and an antibody against neuronal kinesin KIF3A to perform immunological tests on isolated rods. On immunoblots of whole retina and purified rods, anti-LAGSE antibody detected a polypeptide of ~115 kD, anti-Klp1 head detected a polypeptide of ~170 kD, and anti-KIF3A antibody detected a polypeptide of ~80 kD. Indirect immunofluorescence and immunoelectron microscopy with the anti-LAGSE and anti-Klp1 head antibodies localized immunoreactive polypeptides along the length of the connecting cilium and in the RIS. The klps recognized by these antibodies may be involved in transport between the rod inner and outer segments via the connecting cilium. Supported by NIH grant GM 14642 and NSF grant 45147 to JLR.

PHAGOSOME MOTILITY ALONG MICROTUBULES *IN VIVO*
AND *IN VITRO*.

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Using video microscopy of intact cells, we show that internalized latex beads move on linear tracks within cells. This movement is abolished by nocodazole, indicating that it is microtubule-mediated. To better understand this process, we purified phagosomes from J774 macrophages using floatation on a sucrose step gradient, and reconstituted the motility of these organelles along microtubules *in vitro*. For this purpose we have used fluorescent polarity-marked microtubules stabilized with taxol, and fluorescent latex beads. The binding of phagosomes to microtubules requires macrophage cytosol and phagosome membrane proteins (see abstract of Burkhardt *et al.*). Phagosome motility is ATP dependent and occurs 70% towards the minus end of microtubules. The average maximal speed of minus end-directed movement is 1 $\mu\text{m}/\text{sec}$, that of plus end-directed movement 0.8 $\mu\text{m}/\text{sec}$. Approximately 10% of phagosomes move bidirectionally. We are investigating the relative roles of known microtubule motors and accessory factors in controlling the polarity of movement. Since it allows complementary analysis of microtubule binding and motility, this model system provides a simple and practical means to study organelle-microtubule interactions in general.

A FUNCTIONAL REQUIREMENT FOR KINESIN IN THE DROSOPHILA VISUAL SYSTEM.

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Kinesin is a microtubule-associated motor protein that uses energy derived from ATP hydrolysis to translocate along microtubules. In *D. melanogaster*, severe mutations in the kinesin heavy chain gene (*Khc*) result in lethality during the second or third larval instar. Pre-lethal phenotypes of *Khc* mutant larvae indicate that kinesin is a motor for axonal transport and that it is required for normal action potential propagation and neurotransmitter release in motor neurons. To gain insight into the physiological roles of kinesin, we have been utilizing mitotic recombination and clonal analysis to determine the requirement for kinesin function in various adult tissues in *Drosophila*. We have found that stem cells, homozygous for severe *khc* alleles, are capable of founding clonal patches of adult eye tissue. Scanning electron micrographs show that *Khc* mutant patches have a rough phenotype that can be rescued by expressing a transgenic copy of wild type *Khc*. The surface defects include disorganized or missing facets, abnormal lens structures and bristle multiplications. Light microscopy of histological sections of mutant eye tissue reveals a disorganization of the ommatidial array. Individual ommatidia often contain aberrant numbers of photoreceptors. The nature of the mutant patches and the surrounding wild type tissue suggest that *Khc* mutations affect all photoreceptors with equal probability and that the effects of the mutations are cell autonomous. Currently, transmission electron microscopy is being used to search for ultrastructural defects in mutant eye tissue. The identification and characterization of *khc* mutant phenotypes in the developing visual system of both larval and pupal stages are in progress as well.

AXONEME ASSEMBLY IN DROSOPHILA SPERMATOGENESIS
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We are studying the control of microtubule organization, focusing on the role of the *Drosophila melanogaster* gene *whirligig*. *Drosophila* spermatogenesis provides a uniquely well-suited system for elucidating the genetics and biochemistry of metazoan microtubule function, and *whirligig* appears to play a central role in the organization of the sperm flagellar axoneme. *whirligig* (*wrl*) was identified through genetic interactions with mutations in *B2t*, which encodes the testis-specific isoform of β -tubulin. An antimorphic allele of *wrl* enhances *B2t* mutations. Loss of function mutations in *wrl* exhibit dominant male sterility that is suppressed by α - and β -tubulin mutations.¹

Electron micrographs of elongating flagellar axonemes reveal that antimorphic *wrl* mutants lack the accessory microtubules normally associated with the B subfiber of the outer doublet microtubules. In addition, one or both of the central pair of microtubules is often missing. In one loss-of-function *wrl* allele, triplet as well as double microtubules are found in the outer ring. *wrl* mutations appear to have no effect on other microtubule structures either in the testis or in the organism as a whole. *wrl* is thus an excellent candidate for a determinant of axoneme organization in *Drosophila*.¹

To facilitate biochemical and molecular analysis of *wrl* function in axoneme assembly, we are cloning the *wrl* gene by two approaches: (1) positional cloning of DNA tightly linked to *wrl*; and (2) P element transposon mutagenesis of *wrl*. We have identified an 11.2 kb fragment of genomic DNA that, upon germline transformation, restores fertility to male flies heterozygous for both an antimorphic allele of *wrl* and a null mutation in *B2t*. We have also obtained one strong allele of *wrl* by mutagenesis with a P element engineered to facilitate cloning out the flanking genomic DNA. Using these tools, we will clone the *wrl* gene and determine the molecular nature of the gene product. We will express the protein encoded by *wrl* and raise antibodies for immunolocalization experiments to address whether *wrl* acts either at the transition zone between the basal body and the axoneme or along the length of the axoneme to organize its structure.

¹Green, L. L., *et al.* (1990). *Genetics* 126, 961-973.

ACCELERATION OF NUCLEOTIDE EXCHANGE BY PROFILIN COULD CONTRIBUTE TO THE OBSERVED DECREASE IN THE CRITICAL CONCENTRATION OF ACTIN. Michael R. Bubb, David R. Terry, Peter A. Passaro*, Frederick S. Southwick, Daniel L. Purich*, Univ. of Florida College of Medicine, Department of Medicine and *Department of Biochem. and Molecular Biology

Recent studies produced the seminal observation that, under certain conditions, profilin can promote assembly of actin filaments (Pantaloni and Carlier (1993) *Cell*, **75**, 1007). The postulated mechanism was that the interaction of profilin with the barbed filament end is a non-equilibrium process in which ATP hydrolysis is coupled to the addition of the profilin-actin complex, thereby resulting in a lower critical concentration for actin in the presence of profilin. We have obtained data supporting an equilibrium model of interaction between profilin and F-actin, and additionally, have evidence that enzymatic acceleration of the exchange of ATP for ADP bound to monomeric actin contributes to the lower critical concentration of actin. In a solution containing Mg^{2+} -actin filaments at steady-state, monomeric ADP-actin subunits are detected in the absence, but not in the presence of profilin. The percentage of monomeric ADP-actin subunits is independent of total actin concentration, suggesting that filament number, and therefore the loss of ADP-actin subunits from filamentous actin, is not a major contributor to this pool of unpolymerized ADP-actin. In a solution containing Ca^{2+} -actin filaments, the ratio of monomeric ATP-actin to monomeric ADP-actin is much higher than for Mg^{2+} -actin. We believe that the 50-fold higher ATP hydrolysis rates observed for monomeric Mg^{2+} -actin over Ca^{2+} -actin explains why ADP-actin is present under these conditions, although inhibition of actin ATPase activity by profilin (Tobacman and Korn (1982) *J. Biol. Chem.*, **257**, 4166) and dissociation of ADP-actin subunits from filamentous actin may be relevant factors. Because the critical concentration of ATP-actin is lower than that of ADP-actin, the effect of profilin on conversion of ADP to ATP-actin results in a lower critical concentration for Mg^{2+} -actin but not Ca^{2+} -actin. In agreement with this, we find that profilin in the presence of thymosin β_4 promotes polymerization of Mg^{2+} -actin (as previously reported) but not of Ca^{2+} -actin filament assembly.

SIGNAL TRANSDUCTION PROTEINS ASSOCIATED WITH MICROTUBULES RECONSTITUTED *IN VITRO*

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It has been suggested that microtubules (MTs) contribute to signal transduction by direct interaction with members of the signal-transducing G-protein family (*Sakai: Hum. Cell* 4, 93-99, 1991; *Roychowdhury and Rasenick: Biochemistry*, 33, 9800-9805, 1994). Some authors even regard tubulin, the main protein of MTs, as a G-protein because of its significant functional similarity and amino acid sequence homology to the signal transducing G-proteins.

In this study, *in vitro* reconstituted MTs were examined for their ability to bind antibodies against the α -subunit of stimulatory G-protein ($G_{s\alpha}$) and MAP-kinase. Immunoblot analysis of the microtubule protein (MTP), obtained from porcine brain homogenates by three cycles of temperature-dependent MT disassembly/reassembly, containing beside the tubulin about 10-20 % MT-associated proteins (MAPs), revealed three main bands (52 kDa, 67 kDa, 74 kDa) that bound anti- $G_{s\alpha}$ antiserum specifically. These bands correspond to known $G_{s\alpha}$. The anti-MAP-kinase antiserum recognized a characteristic double band at 45 and 52 kDa.

No specific reaction could be detected with anti- $G_{s\alpha}$ in immunoblots of tubulin, separated from MAPs by phosphocellulose column chromatography. With anti-MAP-kinase only the 52-kDa-band was manifested.

Immunocytochemical labelling, detected by fluorescence microscopy using FITC and TRITC-conjugated secondary antibodies, revealed a positive reaction for anti- $G_{s\alpha}$ in the MTs, formed from MTP, over their whole length. The fluorescence pattern of the reaction with anti- $G_{s\alpha}$ could not be distinguished from that obtained with anti- α -tubulin. No immunolabelling was found with anti- $G_{s\alpha}$ in MTs formed from MAP-free tubulin.

Our results indicate a definite copurification and consequently a relatively tight association of $G_{s\alpha}$ as well as of MAP-kinase with MTs, suggesting that these signal transducing proteins are either members of MAP family or bind to the MTs via known MAPs, *i.e.*, MAP1, MAP2, or τ -proteins. Thus, they support the hypothesis on the regulatory role of $G_{s\alpha}$ and MAP-kinase in MT functioning.

MICROTUBULAR SYSTEM IS ESSENTIAL FOR TUBULOGENESIS IN COLLAGEN GELS.

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HGF-SF is a polypeptide identified as a mitogen for cultured hepatocytes and characterized as a factor which enhances the movement and induces the dissociation of the epithelial colonies. HGF-SF is known to induce various types of reorganization in epithelial cultures placed in special conditions. The aim of experiments was to examine the role of microtubular system in HGF-SF induced tubulogenesis in collagen gels with the aid of two microtubule-specific drugs, colcemid and taxol. Aggregates of MDCK cells were cultivated within collagen gels. The cells were incubated with purified recombinant mouse HGF-SF from cell line T3.20.5. (10nM), taxol (2mM), colcemid (0.2 mkg/ml). The microtubular drugs colcemid and taxol were added at early (1 day in culture) and late (7 days in culture) stages of tubulogenesis. Phase contract microscopy was used for the examination of morphological changes induced by HGF-SF. In control conditions, MDCK cells within collagen gels formed rounded compact aggregates. The aggregates treated for 24 hours with HGF-SF acquired polygonal or star-like shapes. As shown by Montesano et al. (1991, Cell, 66: 697) and confirmed by our experiments after 4-6 days of incubation with HGF-SF the cell aggregates were transformed into the systems of branched tube-like structures ending with single elongated cells. Our experiments showed that microtubule-dependent polarization is very important in HGF-SF induced tubulogenesis in collagen gels. Colcemid and taxol completely prevented outgrowth of strands and tubes from three-dimensional cell aggregates. When HGF-SF induces this morphogenesis, the first outgrowths consist of elongated cells oriented along one another. In these conditions, stable polarization of cell shape is essential for the formation of tubes and strands from migrating cells. Thus, microtubular system is essential in complex morphogenesis leading to formation of vectorized multicellular structures.

Author is deeply grateful to Dr. E. Gherardi for a gift of HGF-SF and to Prof. J. M. Vasiliev for generous discussions during the work.

IDENTIFICATION OF A MAIZE cDNA ENCODING A PROTEIN SIMILAR TO ANIMAL CYTOSKELETAL PROTEINS. S.R.Burgess¹ and P.A.Sabelli^{1,2}. ¹IACR-LARS, University of Bristol, UK, ²Present address, IMCB, National University of Singapore, Republic of Singapore.

The development and maintenance of the plant body requires a high degree of coordination in both the root and shoot apical meristems. Higher plants are characterised by their apical localisation of the principal foci of cell proliferation throughout the life span of the plant.

We are using the maize root apex to identify genes which are expressed in proliferation and differentiation dependent manners, and which may have a role in the function and organisation of the root apex.

The maize root apex is physiologically and histologically a well characterised system. There are well defined cell populations in different stages of cell cycle activity, which include the highly active meristem, the quiescent centre, characterised by the low rates of metabolic activity and cell proliferation, and the root cap, a mixture of dividing differentiating and endoreduplicating cells. These different populations of cycling cells have been identified by labelling *in vivo* with ³H thymidine and using *in situ* hybridisation with histone riboprobes.

Differential screening of PCR amplified cDNA libraries constructed from different cell populations of the root apex, have resulted in the isolation of a cDNA, called *ZmCSAP1*, which shows significant similarity to animal proteins such as centrosomin A and caldesmon, which are known to be associated with the cytoskeleton.

Analysis of mRNA distribution using *in situ* hybridisation showed that *ZmCSAP1* is expressed primarily in the central cylinder of the meristem. This specific expression pattern was confirmed by immunofluorescent labelling with a polyclonal antibody raised against a short synthetic peptide, at the level of the light microscope.

This is a novel plant gene with homology to animal cytoskeleton associated proteins. A thorough characterisation of *ZmCSAP1* is under way, including the subcellular localisation of the encoded protein during the cell cycle.

MAP-DEPENDENT BINDING OF PURIFIED PHAGOSOMES TO MICROTUBULES IN VITRO.

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In parallel with our studies of phagosome motility (see abstract by Blocker, et al.), we have investigated the binding of phagosomes to microtubules in a cell-free system. J774 macrophages were allowed to internalize rhodamine latex beads, and bead-containing phagosomes were purified by floatation in a sucrose gradient. Binding of phagosomes to taxol-stabilized rhodamine microtubules was then tested by light microscopy. Binding is stimulated 5-10 fold by incubation in the presence of 2mg/ml macrophage cytosol. At higher concentrations, phagosome binding is inhibited. Order of addition experiments indicate that the binding factor can associate independently with either the membranes or the microtubules. The latter result suggests that the factor is a MAP. Indeed, the activity is lost upon depletion of MAPs from the cytosol, and is detected in a MAP-enriched preparation. Binding is not supported by a preparation of microtubule motor proteins. In addition to cytosolic factors, binding requires the activity of one or more proteins on the phagosome surface. Uninternalized latex beads do not bind, nor do phagosomes which have been treated with certain proteases. Importantly, this microtubule binding machinery appears to be progressively lost as the phagosome becomes more lysosome-like.

THE IDENTIFICATION OF A NOVEL cDNA SEQUENCE EXPRESSED IN BREAST CARCINOMA

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We performed a differential screening of a breast carcinoma cDNA library using multiply subtracted and polymerase chain reaction-amplified cDNA probes in order to identify genes involved in the early stages of tumor progression. A total of 16 genes were identified, including c-erbB2 and tissue inhibitor of metalloproteinases-3, whose products have been implicated in tumorigenesis or invasion, and a novel gene (D52) whose full-length cDNA sequence showed little homology with other nucleotide or amino acid sequences reported in any species. The D52 gene was localized to human chromosome 8q21 using *in situ* hybridization mapping, and its expression was evaluated in human breast carcinomas, non-malignant tissues, and cell lines, using Northern blot and *in situ* hybridization analyses. This indicated that the gene is non-ubiquitously expressed, with D52 transcripts being detected in 8/11 breast carcinomas and 7/7 breast carcinoma cell lines, and using *in situ* hybridization, D52 transcripts were uniquely localized to breast cancer cells within regions of invasive or *in situ* carcinoma in 6/15 breast carcinomas. The mouse D52 homologue was subsequently cloned from a cDNA library constructed from mouse mammary gland tissue undergoing apoptotic involution, and the human and murine coding sequences were found to be highly conserved.

On the basis of features of the murine and human D52 coding sequences, we propose that D52 may function as a novel cytoskeletal or cytoskeleton-associated protein. Further details regarding this hypothesis, and the regulation of D52 gene expression in human cell lines, will be presented.

TARGETING OF PROTEINS TO FUNCTIONAL DOMAINS WITHIN THE MAMMALIAN NUCLEUS

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The mammalian nucleus is highly organized into distinct functional domains separating different biochemical processes and DNA replication is known to occur in discrete patterns of nuclear foci, which undergo characteristic changes throughout S-phase. By the use of multiple labeling immunofluorescence as well as immunoelectron microscopy, replication factors PCNA, DNA polymerase alpha and RPA70 as well as DNA methyltransferase (DNA MTase) have been shown to redistribute within the nucleus and to colocalize with sites of ongoing DNA replication.

We took advantage of this functional organization to analyse regulatory pathways leading to S-phase and to look for a link between cell cycle regulation and DNA replication. We screened for cell cycle proteins present at nuclear replication foci and found cyclin A and cdk2, but not cyclin B1 and cdc2, localized at nuclear replication foci throughout S-phase suggesting a direct role of cyclin A and cdk2 in the control of DNA replication (Cell 74, 979-992). This functional organization may explain how cell cycle kinases have similar biochemical properties in vitro but catalyze specific reactions in vivo.

The biochemical principles of the functional organization of the mammalian nucleus are still largely unknown. In case of the DNA MTase we have, however, identified a distinct protein domain controlling the subnuclear localization (Cell 71, 865-873). This targeting sequence is located within the regulatory domain of the DNA MTase and is dispensable for enzyme activity in vitro, suggesting a regulatory role in the coordination of DNA replication and methylation [4].

These results raised the possibility that such targeting sequences may play a general role in the functional organization of the mammalian nucleus. In support of this hypothesis, we found that human DNA ligase I contains, like DNA MTase, a targeting sequence, which is necessary and sufficient for localization at replication foci. The targeting sequence of the human DNA ligase I is bipartite and has no obvious similarity with the DNA MTase, but is highly conserved between human and mouse. The fact that the targeting sequence is absent in lower eukaryotic and prokaryotic homologues, suggests that 'targeting' is a rather recent development in evolution. These higher levels of organization are likely to contribute to the regulation and coordination of the complex and interdependent biochemical processes in the mammalian nucleus.

FATTY ACID ACYLATION OF TUBULIN

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Tubulin has been localized to several types of membranes including some that are critical for membrane fusion and transduction of extracellular signals. One objection to these studies is that the putative membrane tubulin may have been contaminating cytoplasmic tubulin that coisolated with membrane fractions. An approach to circumvent this objection is to identify a species of tubulin that is membrane-associated, but is biochemically distinct from cytoplasmic tubulin. In most mammalian tissues, multiple tubulin isotypes are generated with unique primary sequences and post-translational modifications. However, neither sequence analysis or known post-translational modifications can account for membrane association. One protein modification that can lead to membrane association is covalent attachment of the long chain fatty acid, palmitate. I investigated the possibility that tubulin is a substrate for palmitoylation. A cell-free system to palmitoylate tubulin was developed. Incubation of porcine brain microtubule protein with rat liver membrane, ^3H -palmitate, Coenzyme A and ATP results in the enzymatic acylation of α - and β -tubulin. ^{14}C -palmitoyl CoA can be substituted for ^3H -palmitate, CoA and ATP. The linkage between palmitate and tubulin appears to be a thioester bond. The substrate for palmitoylation is nonpolymerized tubulin; microtubules are not acylated. Once palmitoylated, tubulin does not assemble into microtubules. Finally, to extend these cell-free studies, I examined palmitoylation of tubulin in platelets. Incubation of human platelets with ^3H -palmitate results in the acylation of α - and β -tubulin as judged by immunoprecipitation and 2D-polyacrylamide gel electrophoresis.

A REPETITIVE ARRAY OF LEUCINES WITHIN THE ACTIN-BINDING DOMAIN IN THE CARBOXY-TERMINUS OF THE BETA SUBUNIT OF CAP Z MAY LINK CAP Z TO THE GELSOLIN/SEVERIN AND PROFILIN FAMILIES OF PROTEINS.

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Previous studies have failed to show significant homologies between Cap Z and proteins from other families of actin-binding proteins. In contrast, within the family of heterodimeric actin-capping proteins, a high degree of identity has been demonstrated in most regions of the protein. Surprisingly, the lowest degree of homology is seen within the only known actin-binding domain of Cap Z, in the carboxy-terminus of the beta subunit. In a recent study of the human Cap Z sequence, we found a motif, KxxxLxxE/DLxxxLxxK/R, that was conserved between the beta subunits of the Cap Z/capping protein family, and also to a variable degree in other actin-binding proteins, including the gelsolin/severin and profilin families of proteins. Within these conserved residues, an array of three regularly spaced leucines was identified. To assess the significance of this finding, we systematically mutated two of these leucines (at position 262 and 266 of the beta subunit of Cap Z), and studied the effects of these mutations on actin-binding. Mutations of leu262 produced proteins with the following order of actin-binding affinities, leu₂₆₂ > ala₂₆₂ > arg₂₆₂, with approximate K_D s of 3.4×10^{-11} , 2.7×10^{-8} and 1.0×10^{-7} , respectively. Mutations of leu266 produced proteins with actin-binding affinities in the order, trp₂₆₆ > ala₂₆₆ > arg₂₆₆ > gly₂₆₆ with approximate K_D s of 3.5×10^{-10} , 4.4×10^{-10} , 2.4×10^{-9} and 3.3×10^{-9} , respectively. The variant proteins all formed heterodimers with the heterologous alpha subunit, and migrated with the same apparent molecular weight on gel-filtration columns, suggesting that their reduced affinity is not due to a global disruption of protein folding.

These studies indicate that the leucines at positions 262 and 266 of the beta subunit of Cap Z are responsible for structure necessary for high affinity capping of actin, and suggest the first possible functional connection between Cap Z and other classes of actin-binding proteins.

THE BACTERIAL ACTIN NUCLEATOR PROTEIN ACTA OF *LISTERIA MONOCYTOGENES* CONTAINS MULTIPLE BINDING SITES FOR HOST MICROFILAMENT PROTEINS

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Several intracellular pathogens, including *Listeria monocytogenes*, use components of the host actin-based cytoskeleton for intracellular movement and cell-to-cell spread. The basic mechanisms underlying the reorganization of the actin-cytoskeleton in this process strongly resemble those required to generate cell motility. The characterization of *L. monocytogenes* mutants unable to accumulate actin filaments led to the identification of the 90kDa surface-bound ActA polypeptide, whose presence is required for both intracellular motility and cell-to-cell spread. When ActA was expressed from an eukaryotic expression vector in transfected cells, it was targeted to the surface of mitochondria, where it induced clouds of filamentous actin demonstrating that it is the sole listerial factor required to initiate recruitment of host actin filaments. Using the mitochondrial targeting assay we have delineated a 23aa N-terminally located sequence on ActA required for actin recruitment. Furthermore, by assessing the ability of various host microfilament proteins to interact with ActA, we have discovered that the Vasodilator-stimulated phosphoprotein VASP, a focal adhesion-associated and profilin-binding protein, binds directly in the absence of other host proteins to the polyproline repeat region of ActA. Deletion of this region from ActA reduces but does not totally abolish actin accumulation, whereas deletion of the actin interacting region abrogates actin recruitment without affecting the ability of the recombinant to bind VASP. Our studies on the listerial ActA polypeptide reveal the initial interactions between this bacterial actin nucleator and host microfilament proteins and suggest that host cell analogues of ActA are important components of structures involved in cell motility.

A DIRECT ACTIN-LIPID INTERACTION CAN MEDIATE
MYOSIN-DRIVEN LIPOSOME MOTILITY *IN VITRO*.

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In order to accomplish its many roles in the cell, actin filaments must inherently be anchored to cell membranes. The study of these attachments has commanded much attention, most of which has focused on membranar actin-binding proteins (mABP). However, we now show that purified actin can bind directly to artificial membranes in the absence of mABPs, and thereby endow lipid vesicles with the ability to undergo myosin-based transport *in vitro*. Using a modified version of Kron's actin-myosin motility assay, we demonstrate that this direct actin-lipid interaction is both necessary and sufficient to permit vesicle motility under physiological buffer conditions. Based on these findings and previous work, we conclude that this novel, direct form of actin-membrane attachment constitutes a functional anchoring mechanism, and is likely to occur *in vivo* in addition to the well-studied mABP-dependent interactions.

**DROSOPHILA MYOSIN LIGHT CHAIN KINASE
ALTERNATIVE SPLICING AND *IN SITU*
HYBRIDIZATION.**

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In the process of finding cell morphogenesis determinants, we have cloned and sequenced a *Drosophila* homologue of the myosin light chain kinase (MLCK) gene. MLCK is known to regulate myosin activity in vertebrate smooth muscle, though its role in striated and non-muscle cells is less clear. We have found that MLCK transcripts are alternatively spliced and that the expected products differ in their regulatory domain, a "pseudosubstrate" that inhibits MLCK activity by obstructing the enzyme's catalytic site. Thus it appears that in *Drosophila*, MLCK activity may be controlled at the level of RNA processing in addition to the known pathways of phosphorylation and calcium regulation.

Embryo *in situ* hybridization, using a probe common to all MLCK transcripts, revealed ubiquitous expression of MLCK in the precellular embryo, the stage at which the actin cytoskeleton is expected to prepare for cellularization. MLCK expression drops after cellularization and remains low in nonmuscle cells. In later stage embryos, intense expression is seen in the striated segmental and pharyngeal muscles. Since the gene encodes a kinase similar to both vertebrate striated and nonmuscle MLCKs, *Drosophila* appears to use a single enzyme to phosphorylate both of its myosin II regulatory light chains. We are currently assessing MLCK isoform distribution in muscle versus nonmuscle cells. Polytene chromosome *in situ* hybridization reveals that the gene lies in a well-characterized chromosomal region (52D) which will facilitate a genetic analysis of its function.

MOLECULAR ANALYSIS OF CENP-E: IDENTIFICATION OF THE KINETOCHORE LOCALIZATION DOMAIN AND OVER-EXPRESSION OF DOMINANT NEGATIVE CENP-E MUTANTS IN CELLS.

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CENP-E is a 312 kd kinesin-like protein that associates with the outer plate or fibrous corona of the kinetochore during mitosis. The N-terminal conserved kinesin motor domain of CENP-E is separated from a second microtubule-binding domain located in the C-terminal region by an extended coil-coil domain of approximately 1700 amino acids.

To identify the domain in CENP-E that target its localization to the kinetochores, various regions of CENP-E were tagged with the green fluorescent protein (GFP). GFP:CENP-E fusion constructs were transfected into Hela cells and localization of the fusion protein was examined in mitotic cells. Using this assay, the C-terminal domain of CENP-E was found to be necessary and sufficient for kinetochore binding. The extreme C-terminal microtubule-binding domain was also found to bundle microtubules in interphase cells.

We also discovered that GFP:CENP-E fusion constructs that specified kinetochore localization during mitosis also block cells in mitosis by acting as dominant negative mutants. An increase in the number of cells with unaligned chromosomes was observed indicating a blockage of mitosis in the transfected cells. In these blocked mitotic cells, the endogenous CENP-E is depleted from the kinetochores and replaced by the GFP:CENP-E fusion proteins. The phenotype of unaligned chromosomes is similar to that observed by microinjection of CENP-E antibodies.

IN VIVO STUDIES OF THE ROLE OF MYOSIN REGULATORY LIGHT PHOSPHORYLATION IN *DICTYOSTELIUM*. Pengxin Chen, Bruce D. Ostrow, Kathleen M. Trybus* and Rex L. Chisholm. Northwestern University Medical School, Chicago, IL. *Rosenstiel Basic Medical Sciences Research Center Center, Brandeis University, Waltham, MA.

Work from a large number of laboratories has shown that the activities of non-muscle and smooth muscle myosins, including that of *Dictyostelium*, are regulated by phosphorylation of regulatory light chain of myosin (RLC). We have employed gene targeting methods to produce *Dictyostelium* cells which fail to express the RLC. These RLC null mutants exhibit defects in cytokinesis and multicellular morphogenesis and myosin localization. We have investigated the importance of RLC phosphorylation in vivo by expressing RLC mutants in which the phosphorylated serine has been mutated to an alanine (S13A) in the RLC null mutants. Myosin purified from these cells carries RLC in the normal 1:1 stoichiometry with the MHC, but the RLC is not phosphorylated. The actin-activated ATPase of this myosin is reduced to levels comparable to those observed for dephosphorylated myosin, indicating that the absence of phosphorylation on the S13A mutant resulted in decreased ATPase activity. However, cells expressing the S13A RLC were indistinguishable from cells expressing wildtype RLC with regard to cytokinesis, development and myosin localization. Thus although phosphorylation regulates the activity of *Dictyostelium* myosin, this regulation is not required for the cells to complete cytokinesis or multicellular development.

The chicken gizzard smooth muscle RLC is also regulated by phosphorylation. It shares about 32 % amino acid sequence identity and 57% similarity to the *Dictyostelium* RLC. We have expressed the chicken gizzard RLC in the *Dictyostelium* RLC null mutant. Expression of the chicken gizzard RLC rescued the cytokinesis and developmental defects of the RLC null mutant. Myosin purified from these cells carried the chicken gizzard RLC in normal 1:1 stoichiometry and did not exhibit significant RLC phosphorylation. In contrast however to the S13A RLC myosin, which had low actin-activated ATPase activity, *Dictyostelium* myosin carrying the chicken gizzard RLC had ATPase activities comparable to myosin carrying phosphorylated wildtype *Dictyostelium* RLC. These results support the idea that the RLC normally represses the activity of *Dictyostelium* myosin, and that phosphorylation relieves that inhibition. It appears that the chicken gizzard RLC is not efficient at repressing the activity of the *Dictyostelium* MHC. Further studies of hybrid RLCs may provide insight into the mechanisms by which RLC phosphorylation regulates myosin activity. Supported by NIH grant GM39264 to RLC.

DEVELOPMENT OF CENTRIPETAL TRACTION FORCE BY CELL SURFACE FIBRONECTIN RECEPTORS. D. Choquet, M. Sheetz, D. Felsenfeld. Dept. of Cell Biology, D.U.M.C. Durham, N.C.

The translocation of adherent cells depends critically on the interactions between the motile cytoskeleton, cell surface receptors and the molecular substrate in the environment. To understand how force is generated on attachment points, we have used laser tweezers to place latex beads coated with the integrin-binding domain of fibronectin on the dorsal surface of the lamellipodia of motile NIH 3T3 fibroblasts. Recombinant fragments of the type III repeats 7-10 of fibronectin (FN) were biotinylated and attached to biotin-BSA coated beads with avidin. The trapping force of the tweezers was used to restrain bead movement and estimate the traction force exerted on the bead by the cell. Different behaviors were observed depending on the concentration of FN on the bead. Control BSA-coated beads displayed little or no binding.

Preliminary experiments have shown specific binding over a wide range of FN concentrations on the beads. With increasing FN concentration, the length of time that the bead diffused in the plane of the membrane before rigid attachment to the cytoskeleton decreased. After attachment, all beads moved toward the rear of the lamellipodia (10-20 $\mu\text{m}/\text{minutes}$). An intermediate behavior consisted of a sum of rearward movement and wobbling with occasional diffusive episodes. At low concentrations of FN, beads came off from the cell surface after 5-60 seconds; while at high concentration they were endocytosed, preferentially at the endoplasm-ectoplasm boundary.

In another paradigm, we kept the laser tweezer on for 10-15 seconds after initial bead-cell contact. Low density FN coated-beads never escaped the trap. As FN concentration increased, beads escaped the trap and moved rearward after shorter intervals. From calibration measurements, we estimated that the cell was pulling with a force greater than 50 pN. Occasionally, at intermediate concentrations of FN, beads would move to the edge of the tweezers and pop back into the center but they would always ultimately escape the tweezers.

In a third series of experiments, we assessed the effect of force (load) on the strength of the cell-bead contact. Different tweezer powers were used to apply different forces to the bead during its movement. Typically, beads would pop back to the center of the tweezers and subsequently overcome the retaining force of the laser trap. Strikingly, the force applied by the cell on the bead increased in proportion to the force applied by the laser trap: a bead could only be recaptured a second time by increasing the power of the trap. On the basis of these findings we suggest that the cell is able to build bead-cytoskeleton contacts to match the load on the bead.

Altogether these experiments suggest that the cell regulate the force exerted on attachment points both as a function of ligand density and load.

**THE STRUCTURE AND FUNCTION OF NOVEL MYOSINS
IN THE NEMATODE, *CAENORHABDITIS ELEGANS*.**
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We are interested in the superfamily of molecular motors known as the myosins. In recent years the bewildering diversity of myosin types and function has become apparent as at least 9 different classes have been identified in a wide variety of organisms. We report the cloning and sequencing of *C. elegans* Myosin 1A, which is, to our knowledge, the first full-length unconventional myosin sequence from this organism. Analysis of the complete cDNA reveals the presence of an open reading frame which encodes a protein of 1022 amino acids with a predicted molecular weight of 117 kD. This protein sequence revealed a conserved motor domain, a single IQ motif and a C-terminal region of ~300 amino acids with a net basic charge but no other recognisable motifs.

We are keen to explore the location and specific function of this unconventional myosin in *C.elegans*. Since the anatomy of the nematode is well understood at the cellular level, including details of all cell-cell contacts at the resolution of the electron microscope, it is an ideal organism for this type of study.

We are in the process of performing developmental Northern blots. The genomic sequence has since been identified and portions of this are being used in a PCR strategy to isolate null mutants.

We have raised polyclonal antibodies to both the motor and tail domains and are using these to isolate the native protein and to conduct immunolocalisation studies. We will identify the light chains which are associated with this myosin and perform biophysical measurements of its motor properties. We will use the confocal microscope precisely to identify the localisation of *C. elegans* Myosin 1A and to elucidate the structures and proteins within the cell to which it associates.

THE ROLE OF CORTICAL GELATION IN SURFACE PROTRUSION

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The cortical actin gel of eukaryotic cells is postulated to control surface protrusive activity. One type of protrusion that may offer clues to this regulation are the spherical aneurysms of the surface membrane known as blebs. Blebs are thought to be fluid-driven protrusions and occur normally in cells during spreading, where they alternate with other protrusions such as ruffles, suggesting similar protrusive machinery is involved. We recently reported prolonged blebbing in human melanoma cell lines deficient in the actin filament crosslinking protein, ABP-280, which offer a good system to study this protrusion. The blebs expand at different rates of volume increase that directly predict the final size achieved by each bleb, but the rates decrease as the intracellular F-actin content of the cells increases over time. This decrease in rates occurs much more rapidly, and at a lower concentration of intracellular F-actin, in ABP-280 expressing cells. Fluorescently-labeled actin and phalloidin injections of blebbing cells indicate that a polymerized actin structure is not present initially, but appears later and is responsible for stopping further bleb expansion. As increasing actin gelation is associated with increased impedance to fluid flow, it is postulated that the decrease in cortical gelation seen in these cells allow a fluid-driven expansion of the cell membrane sufficiently rapid to initially outpace the local rate of actin polymerization, and blebbing results. The rate of this solvent flow decreases as gelation is achieved, whether by ABP-280, or other gelation factors, and leads to decreased size and occurrence of blebs. Since these forces driving bleb extension are always present in a cell, this process may influence other cell protrusions as well.

MEMBRANE-CYTOSKELETAL TARGETING POTENTIAL OF PROTEIN DOMAINS PRESENT IN PROTEIN-TYROSINE PHOSPHATASE PTP-BL.

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Phosphorylation on tyrosine residues at submembranous regions in the cell does not only have a function in transporting growth and differentiation signals from the membrane to the nucleus. The integrity of many junctional structures are also depending on the tyrosine phosphorylation status of their components.

Recently, we have cloned a murine protein tyrosine phosphatase (PTP-BL) that is expressed in epithelial cells and displays intriguing sequence homologies with the *neurofibromatosis type 2* and *Drosophila Expanded* and *Discs-large* tumor suppressors.

The presence of a band 4.1-like domain and five discs-large homologous regions (DHRs) in PTP-BL suggest a submembranous junctional localization in epithelial cells, and its catalytic tyrosine dephosphorylating potential implies an important role in morphology and motility of epithelia.

We have tested the targeting potential of the various PTP-BL protein domains by transient transfection experiments in epithelial cells. Co-localization studies using confocal laser scanning microscopy and immuno-electron microscopy revealed the ability for the PTP-BL band 4.1-like sequence to target the protein to the cytoplasmic side of the cell surface membrane. Similar experiments are now performed to test the routing potential of the five PTP-BL DHR motifs, and preliminary data suggest a more general association to cytoplasmic faces of (intra-)cellular membranes.

MOLECULAR ANALYSIS OF A GELSOLIN-FAMILY MEMBER ENCODED BY THE *FLIGHTLESS1* GENE OF *DROSOPHILA MELANOGASTER*.

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The flightless gene of *D. melanogaster* encodes a novel member of the growing family of putative actin-severing and nucleating proteins, which consist of leucine-rich repeats and gelsolin-like segments (de Couet, *et al.*, 1995, Genetics, submitted). Homologous genes with more than 50% similarity have also been identified in nematode worms and humans (Campbell, *et al.*, 1993. PNAS 90: 11386-11389). Our detailed molecular and cell biological analysis has involved mapping the molecular lesions affecting two embryonic lethal and three viable alleles and evaluation of mutant forms of its translation product relative to the crystallographic structure of the G1 domain of gelsolin.

Comparison with gelsolin revealed that sidechains important for the structural integrity of the hydrophobic core of G1 are fully conserved in the homologous domain of flightless. Many of the residues involved in the contact surface with actin are also conserved in the flightless protein. We sequenced the entire wild-type flightless genomic DNA landscape, and by sequencing the viable flightless alleles determined that two of these encode mutant proteins exhibiting amino acid replacements within the F1 domain. These mutations result in conservative amino acid replacements affecting a region of F1 with putative calcium-binding properties in G1 of gelsolin. The phenotype of these mutants appears to be restricted to the indirect flight muscles, whereas lethal alleles affect cellularisation and gastrulation (Miklos & de Couet, 1990, J. Neurogenetics 6: 133-151). The third viable flightless mutation affected the leucine-rich repeat domain. An additional lethal mutation has a deletion extending from the first intron upstream to the promoter region, and includes the translational start site. We discuss our results in the light of the phenotypic characteristics of the mutants, the putative functions of the protein and compare our findings to the molecular defect observed in the mutant gelsolin gene characterizing the human disease Finnish Familial Amyloidosis.

Preliminary antibody studies with the flightless gene product indicate that it is associated with the Z-band of both indirect flight muscles and visceral muscles. We argue that the leucine-rich repeat domain of flightless may be responsible for the subcellular targeting of the protein to these structures.

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GENETIC INTERACTIONS BETWEEN THE *DROSOPHILA* PROFILIN GENE (*CHICKADEE*) AND EGF RECEPTOR SIGNAL TRANSDUCTION CASCADE COMPONENTS.

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We report here genetic evidence linking the actin and PIP₂ binding protein profilin to a receptor tyrosine kinase (RTK) signal transduction pathway. We have characterized a dominant allele of *chickadee*, *chic^{gdh-5}*, that causes ectopic wing crossveins in 24% of heterozygous adults. The penetrance of the ectopic crossvein phenotype in combination with various loss of function alleles of *chic* suggests that *chic^{gdh-5}* acts as a competitive inhibitor of wild-type profilin. We have also characterized a new P-element allele, *chic^{K66/20}*, with phenotypes similar to *chic^{gdh-5}*.

Mutations in components of the *Drosophila* EGF receptor (*EGFr*) signalling pathway dominantly effect penetrance of the crossvein phenotype, consistent with previous *in vitro* studies that have implicated profilin as a component of RTK signalling pathways. The *EGFr* gain-of-function mutation *Ellipse* increases the penetrance of extra crossveins in *chic^{gdh-5}/Elp* heterozygotes to 62%, and to 86% in *chic^{gdh-5}, Elp/Elp* adults, while *EGFr* loss-of-function mutations reduce penetrance to less than 10%. Loss of function mutations in *Ras1* dominantly suppress penetrance to less than 5%, and *Ras1^{val12}* (which produces a constitutively active form of ras) dominantly enhances penetrance to 51%.

Using a genetic screen for dominant modifying mutations that interact with *chic^{gdh-5}*, we have identified seven additional loci and six deficiencies that dominantly enhance or suppress the crossvein phenotype, and may interact directly or in common signalling pathways with profilin.

The profilin encoded by *chic^{gdh-5}* acts as an antimorph, that is, an inhibitor (in a genetic sense) of wild-type profilin. Based on current models for the biochemical activities of profilin, we hypothesize that the mutant profilin might bind to monomeric actin but not catalyze nucleotide exchange, or might compete with wild-type profilin for binding to phosphoinositides but not inhibit phospholipase activity.

We hypothesize that profilin is directly involved in a signalling pathway activated by *EGFr* to induce crossvein formation in the wing. Since this pathway might be used for signalling processes elsewhere in the developing fly, genetic interactions in the wing may be useful for elucidating connections between the cytoskeleton and RTK signalling in other developmental processes.

ACTIN FILAMENTS AND MYOSINS I AS REGULATORS OF RECEPTOR MEDIATED ENDOCYTOSIS.

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Recent reports have pointed out the role of actin filaments for the early steps of apical endocytosis in polarized epithelial cells (Gottlieb et al., J. Cell Biol. 1993, 120, 695-710; Jackman et al., J. Cell Sci 1994, 107, 2547-2556). In order to investigate whether actin filaments are also involved in receptor mediated endocytosis in non polarized cells, we have studied the endocytic pathways of transferrin and $\alpha 2$ -macroglobulin in an hepatoma cell line (BWTG3). Towards this goal, 2 different strategies have been developed. The first one uses a Cytochalasin D treatment ($1\mu\text{m}$) that depolymerized actin filaments and the other one involves the production of an actin based motor protein, the brush border myosin I (BBMI) and 2 of its truncated variants deleted in the motor domain (BBMI Δ 40 and BBMI tail). We show that Cytochalasin D: 1) impairs the recruitment, at one pole of the nucleus, of the two ligands after 30 min of internalisation 2) decreases by 3 fold the kinetic of transferrin uptake 3) inhibits the rate of $\alpha 2$ -macroglobulin degradation up to 60% and 4) also impairs the transport of ligands (accumulated in late endosomes in cells incubated at 18°C and then warmed up to 37°C) from late endosomes to the plasma membrane or to the degradative compartments. On the other hand, the production of the truncated variants of BBMI impairs the distribution of: 1) the juxtanuclear recycling compartment of internalized transferrin, 2) the late endocytic compartment ($\alpha 2$ -macroglobulin internalised for 30 min), 3) lysosomes and 4) the acidic compartment. The rate of alpha 2-macroglobulin degradation was increased by 80% and 45% over control cells, in cells producing BBMI Δ 40 and BBMI tail respectively. Furthermore, these variants codistribute with the late endocytic compartment characterized by the distribution of $\alpha 2$ -macroglobulin internalized for 30min. Altogether, these results indicate that the integrity of actin filaments is required for 2 steps along the endocytic pathway. The first one, from the plasma membrane to endosomes and the second one, from late endosomes to the degradative compartments. Furthermore, they also suggest that an acto-myosin mechanism is involved to maintain the structure of the late endocytic compartment and regulate the delivery of ligands from the late endocytic compartments to the degradative compartments. Our findings raise the question of cooperative actions between microtubules and actin filaments in membrane trafficking. The role of the first one is well documented whereas the contribution of the second one has remained elusive so far.

**RESTORATION OF ACTIN CYTOSKELETON AT VARIOUS
LEVELS OF P-GLYCOPROTEIN-MEDIATED MULTIDRUG
RESISTANCE (MDR)**

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P-glycoprotein (Pgp) is an efflux pump excluding many toxic drugs from the cell. We have studied actin cytoskeleton in the hamster cells with various levels of Pgp-mediated MDR (from 10-fold to 40 000-fold colchicine (CH) resistance) and various levels of Pgp activity. Our major result is the tendency to partial restoration of actin stress fibers in the cells with initial level of MDR and complete restoration of stress fibers in the cells with high MDR level. Parental malignant cells contained actin in dispersed form, as determined by rhodamine-phalloidin staining. In contrast, in 10-fold resistant sublines up to 30% cells demonstrated stress-fibers. Stress fibers were restored in all cells with 42600-fold CH-resistance. Both microtubules and intermediate filaments did not alter in drug-resistant cells. They formed disordered network both in drug-sensitive and -resistant cells. It was shown that Pgp activity is regulated through protein kinase C (PKC) pathway (Chaudhary, Roninson, 1992). Our preliminary data show that PKC function (phosphatidylcholine synthesis) is altered in the cells with Pgp hyperfunction. It is possible that actin stress fibers restoration in MDR cells is due to alterations in signaling pathway.

CYTOSKELETAL COMPONENTS AND THE LOCAL MODULATION OF AXON CALIBER BY MYELINATING SCHWANN CELLS.

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When maturing axons are invested with neurofilaments, their diameter may increase by more than an order of magnitude. Further augmentation of axon caliber is observed as axons become ensheathed and myelinated. The latter change correlates with the expression of novel phosphorylation epitopes on cytoskeletal elements. Despite the close association of such post-translational modifications and local axon caliber, it is presently unknown to what extent, if any, different cytoskeletal elements contribute in the axonal response to myelin. Here, in an effort to determine the relative role of neurofilaments and non-neurofilament components in this response, we have measured the caliber of axons with and without a neurofilament cytoskeleton in both myelinated and amyelinated regions.

In transgenic mice expressing an NFH-lacZ fusion gene, the endogenous neurofilament proteins assemble into neurofilaments. However, in the presence of the fusion protein, neurofilaments become crosslinked in the neuronal cell body and precipitate into large aggregates. As a consequence, they are unavailable for export and most axons in these mice are markedly deficient or entirely devoid of neurofilaments. In mice homozygous for the *dystrophia musculorum* (*dy*) allele, Schwann cells fail to ensheath many of the axons in the longer spinal roots. *dy/dy* mice were crossed to the NFH-lacZ transgenic mice and in subsequent generations, affected *dy/dy* mice, with and without the transgene, were recovered. Their spinal roots were processed for electron microscopy and the axon calibers achieved in myelinated and amyelinated were compared.

Results to date clearly confirm the importance of axonal neurofilaments in the establishment of large axon calibers. With or without myelin, axons invested with neurofilaments were markedly larger than those without neurofilaments. However, comparisons between myelinated and naked axon populations demonstrated that even the neurofilament deficient axons achieved larger calibers when myelinated. Therefore, components other than neurofilaments appear to participate in the local response of axons to myelination. We are presently investigating a larger axon sample with the aim of establishing the absolute extent of the caliber change mediated by non-neurofilament components.

MITOTIC DEFECTS IN *DROSOPHILA GLUED*¹

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Drosophila *Glued* is a homolog of the p150*Glued*/dynactin subunit (1), which has been shown to regulate dynein microtubule motor activity in vitro (2). Microtubule motor mutants in yeast and fungi show defects in spindle assembly and chromosome segregation during mitosis (3); in higher eukaryotic cells, the role of microtubule motors in mitosis is still unclear. *Glued*¹ is a dominant mutant which causes defects in the development of the compound eye and its connection to the optic lobes (4). Molecular analysis has shown the mutation is caused by a transposon insertion which truncates the 3'-end of the transcript (5). Genetic analysis suggests the mutant protein poisons the dynactin complex (6).

We have found mitotic defects in developing *Glued*¹ eyes. Nearly twice as many dividing cells are found in the second mitotic wave of mutant third instar eye discs. The distribution of mitotic stages is altered in *Glued*¹ eyes; large numbers of dividing cells arrest in metaphase. In order to study *Glued*¹ mitotic defects more closely, we looked at mitosis in the syncytial blastoderm of mutant embryos. Immunostaining using β -tubulin antibody reveals mitotic spindle defects, including missing astral microtubules and barrel-shaped spindles. Hoechst staining shows chromosome segregation is abnormal in mutant embryos. In wild type embryos, cell division is tightly synchronized; in *Glued*¹ however, a small population of asynchronous divisions occurs. We have made a polyclonal antiserum against a bacterial fusion protein which encodes an N-terminal *Glued* polypeptide. This antiserum localizes p150*Glued* to mitotic spindles and spindle poles during mitosis. These results provide the first evidence that p150*Glued* is involved in mitotic spindle assembly and chromosome segregation during mitosis.

(1) Holzbaur et al. 1991. *Nature* 351:579. (2) Gill et al. 1991. *J. Cell Biol.* 115:1639. (3) Muhua et al. 1994. *Cell* 78:669. Plamann et al. 1994. *J. Cell Biol.* 127:139. Li et al. 1993 PNAS 90:10096. Eshel et al. 1993. PNAS 90:11172. Saunders et al. 1995 *J. Cell Biol.* 128:617. (4) Meyerowitz and Kankel 1978. *Dev. Biol.* 62:112. (5) Swaroop et al. 1987. *PNAS* 84: 6501. (6) Harte et al. 1982. *Genetics* 101: 477.

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STOCHASTIC DYNAMICS OF MICROTUBULES: A MODEL FOR CAPS AND CATASTROPHES

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We have formulated and solved a phenomenological model for the so-called *catastrophes*, the abrupt transitions from the growing to the shrinking state of microtubules. The model may explain existing experimental results and resolve some long-standing apparent contradictions. In particular, the model reproduces observed catastrophe rates, see Fig. 1, and waiting times for catastrophes upon sudden dilution, see Fig. 2. It may also explain why recent experiments fail to measure the GTP content in growing microtubules and provides a mechanism for so-called *coupled hydrolysis*. Details are given in [1].

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- [2] D. N. Drechsel, A. A. Hyman, M. H. Cobb, and M. W. Kirschner, Mol. Biol. Cell **3**, 1141 (1992).
- [3] D. Kuchnir Fygenson, E. Braun, and A. Libchaber, Phys. Rev. E **50**, 1579 (1994).
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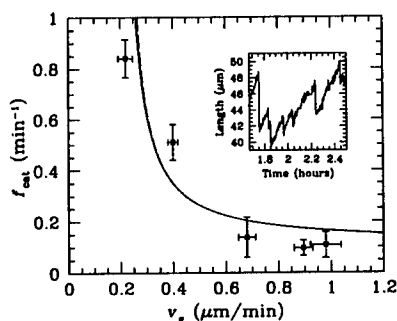


FIG. 1. Catastrophe rate, f_{cat} , versus growth velocity, v_g . Dots with error bars: experimental results [2]. Full and dashed curves: catastrophe rates derived in [1] from 3-parameter theory which was fitted simultaneously to this and the next figures experimental data. Inset: length as a function of time for a single microtubule. Data was taken from [3].

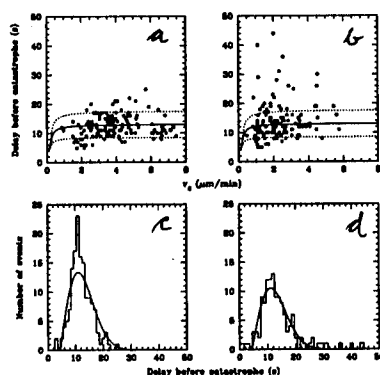


FIG. 2. Delays before catastrophe induced by dilution. Data was taken from [4]. (a) and (c): plus end; (b) and (d): minus end. (a) and (b): delay as a function of initial growth velocity. Each point represents a single measurement on a microtubule. Curves are theoretical mean (solid) and standard deviation (dashed) of the delay derived in [1]. (c) and (d): histograms showing the experimental distribution of delays before catastrophe. The curves are theoretical distributions derived in [1].

THE DROSOPHILA MATERNAL-EFFECT MUTATION GRAPES
DISRUPTS MIDBODY FORMATION AND PRODUCES A METAPHASE
ARREST AT NUCLEAR CYCLE 13

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grapes (grp) is a second chromosome (36A-B) maternal-effect lethal mutation in *Drosophila melanogaster*. We demonstrate that the syncytial nuclear divisions of *grp*-derived embryos are normal through metaphase of nuclear cycle 12. However, as the embryos progress into telophase of cycle 12, the microtubule structures rapidly deteriorate and midbodies never form. Immediately following the failed midbody formation, sister telophase products collide and form large tetraploid nuclei. These observations suggest that the function of the midbody in the syncytial embryo is to maintain separation of sister nuclei during telophase of the cortical divisions. After an abbreviated nuclear cycle 13 interphase, these polyploid nuclei progress through prophase and arrest in metaphase. The spindles associated with the arrested nuclei are stable for hours even though the microtubules are rapidly turning over. The nuclear cycle 13 anaphase separation of sister chromatids never occurs and the chromosomes, still encompassed by spindles, assume a telophase conformation. Eventually neighboring arrested spindles begin to associate and form large clusters of spindles and nuclei. To determine whether this arrest was the result of a disruption in normal developmental events that occur at this time, both *grp*-derived and wild-type embryos were exposed to X-irradiation. Syncytial wild-type embryos exhibit a high division error rate, but not a nuclear-cycle arrest after exposure to low doses of X-irradiation. In contrast, *grp*-derived embryos exhibit a metaphase arrest in response to equivalent doses of X-irradiation. This arrest can be induced even in the early syncytial divisions prior to nuclear migration. These results suggest that the nuclear cycle 13 metaphase arrest of unexposed *grp*-derived embryos is independent of the division-cycle transitions that also occur at this stage. Instead, it may be the result of a previously unidentified feedback mechanism. Molecular analysis indicates that *grp* shares extensive homology to the *S. pombe* checkpoint kinase gene, *chk1/rad27*.

MAMMALIAN SKELETAL MUSCLE MYOMESIN: BIOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF A PKC-SITE

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The cytoskeleton of vertebrate striated muscle myofibrils is composed of titin and several associated proteins. In the center of the sarcomere, i.e. in the M band region, titin is bound to myomesin and M-protein, two proteins that earlier have been described as myosin-binding. Molecular cloning identified all sarcomere cytoskeletal proteins as intracellular members of the immunoglobulin superfamily.

We have now for the first time succeeded in purifying myomesin from bovine skeletal muscle to homogeneity. This allowed a thorough biochemical characterization of the polypeptide. The identity of the protein was confirmed by its reactivity with myomesin-specific monoclonal antibodies as well as polyclonal antibodies directed against internal peptides derived from the translated human cDNA. Analytical ultracentrifugation and circular dichroism spectra confirmed the strong similarity to M-protein predicted from sequence comparisons. In a dot-blot overlay-assay purified myomesin specifically bound to myosin-rod and LMM, while a C-terminal 30 kDa fragment of LMM was not decorated. Proteolytic digests and bacterially expressed domains were used to show that this myosin-binding site is located in the first two immunoglobulin domains of myomesin.

The crossreactivity with a phosphorylation-dependent neurofilament antibody allowed us to identify a PKC-phosphorylation-site at the C-terminus of myomesin. This is a first hint that myomesin could be involved in a signal transduction pathway that regulates the assembly of the myofibril.

TETRAHYMENA BASAL BODY PROTEINS NUCLEATE ACTIN
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In *Tetrahymena*, microfilaments are integrated with basal bodies through the association of these organelles with a fibrillar cage which surrounds each basal body. The cage is connected to triplet microtubules by actin filaments (*J. Cell Sci.* **103**: 629-641, 1992) which are closely associated with a myosin II-like protein (*J. Cell Sci.* **108**, no. 3, 1995). In the present report, we have focused on the nucleation of actin filaments by basal body proteins as a possible mechanism for the integration of actin filaments with basal body microtubules in the cage complex.

The cartwheel is an intermediate in the formation of basal bodies. Basal body proteins in microtubule reassembly buffer will form cartwheels and dense aggregates which may contain cartwheel components but do not display the characteristic cartwheel morphology (*J. Cell Sci.* **66**: 147-154, 1984). For the present study, we have tested the nucleation potential of this reassembled material.

Reassembly pellets were resuspended in actin polymerization buffer and mixed with skeletal muscle G-actin. Phalloidin was added to enhance polymerization and to stabilize newly polymerized filaments. The actin was allowed to polymerize, and the mixture was then centrifuged at 10,000 *g* which sedimented the reassembled material but did not sediment single actin filaments. Thin sections of the reassembled material were labeled with an anti-actin antibody followed by secondary antibody linked to 5 nm colloidal gold particles. Electron microscopy showed that colloidal gold particles decorated actin filaments which emerged from the dense aggregated material but did not decorate the reassembled cartwheels. In a control experiment, reassembled material was mixed with pre-assembled actin filaments in actin polymerization buffer and sedimented at 10,000 *g*. Labeling of the reassembly aggregates by the anti-actin antibody was drastically reduced in the control preparations.

In order to show that the labeled actin filaments were derived from the polymerization of exogenous G-actin and were not the result of a redistribution of endogenous F-actin, reassembly material was labeled with FITC-phalloidin which binds to vertebrate F-actin but does not bind to *Tetrahymena* actin. The reassembly aggregates were brightly fluorescent in samples that had received G-actin, but they showed only background-level fluorescence in control samples.

These results demonstrate that actin filaments can assemble onto aggregated basal body proteins which could function as stable nucleating sites for actin filaments during the morphogenesis of the cage complex. (Supported by NSF Grant MCB 930194)

INTERDEPENDENT PHOSPHORYLATION SWITCHES ARE SPECIFIC FOR THE CARDIAC ISOFORM OF MYOSIN-BINDING PROTEIN C

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Cardiac myosin-binding protein C (cardiac MyBP-C, cardiac C-protein) belongs to a family of sarkomeric proteins implicated in both regulatory and structural functions of striated muscle. For the cardiac isoform, regulatory phosphorylation in vivo by cAMP-dependent protein kinase (PKA) upon adrenergic stimulation is linked to modulation of cardiac contraction. Binding of the MyBP-C protein family to both myosin and titin had previously been demonstrated by several laboratories.

In the course of the characterization of titin ligands, we have determined the complete cDNA sequence of human cardiac MyBP-C which reveals regulatory motifs specific for this isoform. Phosphorylation is restricted to the N-terminal region of the native molecule as well as to recombinant N-terminal fragments. Site-directed mutagenesis identifies an RRIS motif in a cardiac-specific loop insertion in the N-terminal region of cardiac MyBP-C as the key substrate site for phosphorylation by both PKA and a calmodulin-dependent protein kinase associated with the native protein. Phosphorylation of two further sites by PKA is induced by phosphorylation of this isoform-specific site. This phosphorylation-induced switch can also be mimicked by aspartic acid instead of phosphoserine. Cardiac MyBP-C therefore seems specifically equipped with sensors for adrenergic regulation of cardiac contraction, possibly implicating cardiac MyBP-C in cardiac disease. A second cardiac-specific 38 residue insertion in an IgI-domain shows resemblance to SH3-binding sequences.

A NOVEL METHOD TO ASSESS THE FORM OF CILIARY BEATING IN CULTURED MUCOCILARY EPITELIUM.

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Cilia are cellular protrusions which transport mucus in digestive, reproductive and respiratory systems of vertebrates. Normal mucociliary activity is essential for the regular function of the systems in which the cilia are found. Ciliary beating is characterized by high degree of temporal and spatial asymmetry. The fast effective stroke, during which the propulsion of the mucus occurs, is performed in a plane perpendicular to the cell surface. It is followed by a slow recovery stroke, during which cilia return to their initial position moving in a plane inclined to the cell surface. Sometimes a pause separates the two strokes.

Although the characteristics of the beat form influence the efficiency of mucus propulsion, due to lack of appropriate and easy techniques, limited data are available, about the dependence of the beat form on experimental conditions. In the present work we demonstrate a novel photoelectric method to measure the ciliary beat in an easy, non destructive and real-time manner.

The suggested method utilizes the photoelectric signals which are obtained by optic fibers monitoring of the light transmitted through a cultured active ciliary area. These signals contain all the information about the ciliary movement in the observed area. We have simulated photoelectric signals which emulate the amount of transmitted light through a ciliary epithelium. The shape of the artificial signals was found to depend on the parameters of the simulated ciliary beating. The comparison between the computer-generated signals and the measured photoelectric signals provided us with information about ciliary beating parameters during the measurement. The number of free parameters in the simulations was reduced by using the measurement of the delay times between cilia in two directions by the three-point-simultaneous technique¹, and the mathematical relations between the times of delay and the cilium beating parameters².

With the aid the suggested technique we characterized the form of ciliary beating in cultured frog esophagus under normal conditions and as a function of ciliary stimulation by extracellular ATP.

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2. Gheber, L., and Priel, Z. 1990 *Cell Motility and the Cytoskeleton*. 16:167-181.

FUNCTIONAL ANALYSIS OF PROFILIN-LIGAND INTERACTIONS

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We have investigated the interaction of profilins with actin and with poly-proline motif containing ligands. Recombinant profilins from bovine and birch origin, and a chimaeric protein containing the actin binding domain of Dictyostelium α -actinin fused N-terminally to bovine profilin were used to characterize in vitro the modes of interaction of profilin with G- and F-actin, and to compare these in homologous and heterologous systems.

In addition, we have identified the first biological ligands exploiting the poly-L-proline binding motif present in animal and plant profilins. The poly-proline rich protein VASP was previously identified as a substrate for cAMP- and cGMP-dependent protein kinases in platelets and as an F-actin-binding component of focal contacts. VASP and additional proteins present in calf thymus and human platelets were found to interact in vitro specifically through poly-proline-rich sequences with both types of profilins.

In addition, we have analyzed the association of profilin with actin and VASP in living cells under various conditions. Our results demonstrate the following: (1) Animal and plant profilins can functionally substitute for each other in the organization of animal microfilaments, in the cortical actin network as well as in newly formed stress fibers. (2) Overexpression of profilin protects actin filaments against stress agents. (3) Transformed cells contain a higher ratio of profilin to actin than normal cells, as seen in quantitative immunoblots with specific antibodies against profilin and actin, respectively. (4) VASP colocalizes with profilin in dynamic areas of spreading cells.

These studies emphasize the highly conserved role of profilin as a potent regulator of microfilament organization and suggest that profilins themselves are probably subject to regulation by kinase-mediated signal transduction pathways.

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PLATELET ADDUCIN: INDUCTION OF A SPECIFIC ISOFORM IN K562 CELLS

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Adducin is an actin and spectrin crosslinking protein originally described in the membrane skeleton of erythrocytes. Red blood cell (RBC) adducin is a tetramer composed of two very similar polypeptides encoded by distinct genes (α - and β -adducin). From immunoblotting tissues and cell lines it is now apparent that adducin gene products are expressed in a wide variety of cells and several cDNAs have been described encoding alternatively spliced isoforms from both genes. Human platelets examined by immunoblotting showed a band which comigrates with α 1 adducin (the RBC isoform) and a band with relative mobility of approximately 65,000 daltons which has not been described previously. Using newly developed antibodies which are specific for α - or β -adducin gene products, the 65 kd band in platelets appears to be derived from the β -adducin gene.

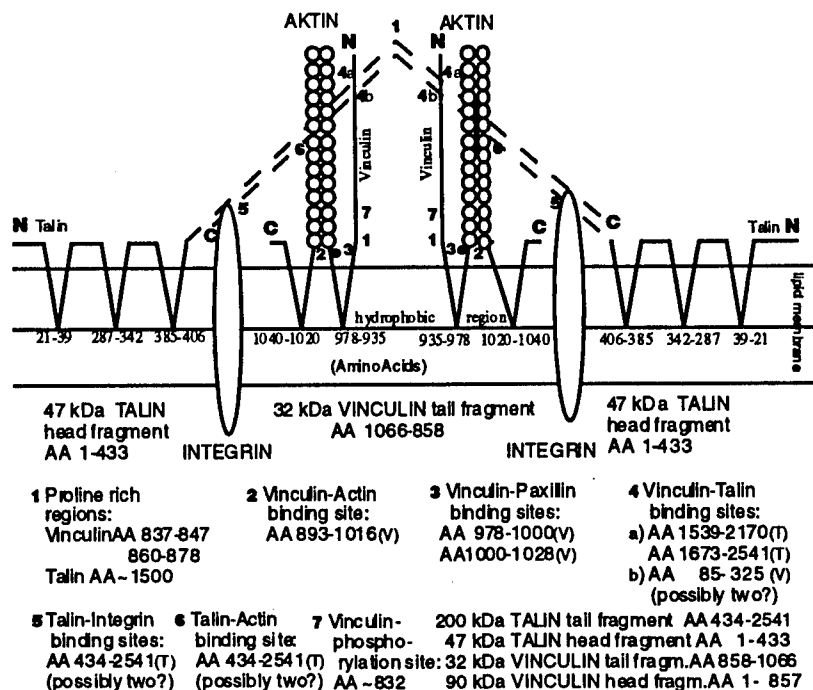
K562 is a human erythroleukemia cell line used as a model for studying differentiation along erythroid (RBC) versus megakaryocyte (platelet) lineages. Upon treatment with phorbol esters such as TPA, K562 cells undergo a phenotypic transition characterized by expression of several megakaryocyte specific proteins. Using this cell model, K562 cells were treated with TPA and showed marked induction of the 65 kd platelet isoform of β -adducin. Expression of the 65 kd isoform of β -adducin appears to be associated with differentiation along the megakaryocytic pathway in this model system. The 65 kd isoform of adducin is likely to have molecular interactions which differ from RBC adducin, suggesting that platelet adducin may have platelet-specific functions.

COMPUTER EVALUATION AND MODEL PREDICTION OF THE INTERACTION OF TALIN/VINCULIN WITH LIPID MEMBRANES.

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Recent *in vitro* experiments showed that the 47kDa talin and 32kDa vinculin fragment interact with acidic phospholipids. We determined by computer analysis the hydrophobic and amphipatic stretches of these fragments and by applying a purpose-written matrix we ascertained the molecular amphipatic structure of α -helices. Calculations for the 47kDa mouse talin fragment (residues 1-433; N-terminal region) suggest specific interactions of residues 21-39, 287-342 and 385-406 with acidic phospholipids and a general lipid-binding domain for mouse talin (primary sequence residues 385-401) and for Dictyostelium talin (primary sequence residues 348-364). Calculations for the 32kDa chicken embryo vinculin fragment (residues 858-1066; C-terminal region) and results from nematode vinculin alignment indicate for chicken embryo vinculin (residues 935-978 and 1020-1040) interactions with acidic phospholipids.

A model deduced from experimental/theoretical data.



Possible Influence of Nebulin on the Gelsolin-Sensitivity of Thin Myofilaments

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In permeabilized, cross-striated chicken myotubes in culture, thin filaments are resistant against the severing activity of gelsolin (in contrast to isolated myofibrils, cf. Funatsu et al., J. Cell Biol. 110, 53-62, 1990). Using immunofluorescence after incubation with gelsolin, it was localized specifically in the thin filament regions of these cells. No significant extraction of filamentous actin was detected, whereas non-myofibrillar actin was completely removed. To investigate the reason for this stability, permeabilized myotubes were preextracted with a high ionic strength buffer to eliminate tropomyosin and myosin as possible stabilizing factors, and then gelsolin was added. Again, a specific localization in the I-Z-I-region was observed indicating binding to thin filaments without severing, since the actin pattern after rhodamine-phalloidin staining remained unchanged. The distribution of the external gelsolin was identical to that of endogenous gelsolin. Because of these observations, it was assumed that nebulin, a skeletal muscle-specific giant protein, might be responsible for the gelsolin-resistance of sarcomeric actin filaments. In fact, nebulin was detected by immunofluorescence microscopy after both extraction with Triton X-100 and with high salt buffer. Since nebulin was the only residual thin filament component in the extracted cells we regard it likely that this protein causes gelsolin-resistance of thin filaments in skeletal myotubes. This assumption was corroborated by the fact that - even without high salt preextraction - cardiac myocytes (which do not contain nebulin) were fully sensitive to gelsolin.

Therefore we investigated the severing action of gelsolin on chicken cardiac myocytes which had been pre-incubated with a 6 repeat (~6x35 aa) - recombinant nebulin fragment (SR 635-6636, Labeit et al., FEBS Lett. 282, 313-316, 1991). In vitro, sedimentation assays revealed a specific binding of this fragment to F-actin. Also in situ, binding of the nebulin fragment was demonstrated by double-immunofluorescence of cardiac cells with anti-nebulin and rhodamine-phalloidin. After incubation of the permeabilized cardiac cells with an excess of SR 635-6636 and subsequent treatment with gelsolin, a removal of the sarcomeric thin filaments was observed though nebulin was still detected in the I-Z-I-region of the sarcomers.

These results show that binding of the recombinant nebulin fragment to thin filaments in cardiac myocytes is possible but this interaction is apparently not sufficient to prevent severing by gelsolin. Either the binding is incomplete or the fragment is too short for a stable binding since it lacks one repeat to a complete super-repeat of intact nebulin.

FUNCTIONAL DOMAINS WITHIN THE ALTERNATIVELY SPLICED NINTH EXON OF TROPOMYOSIN

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Only exons 2 and 9 differ between smooth and striated muscle α -TMs. The striated exon 9 is only expressed in striated muscle and encodes part of the TnT binding site required for Tn-linked regulation, found only in striated muscles (Zot and Potter. 1987. *Annu. Rev. Biophys. Biophys. Chem.* 16:535). The smooth exon 9 is found in smooth muscle and nonmuscle cells (Lees-Miller et al. 1990. *Mol. Cell Biol.* 10:1729). Bacterially produced recombinant striated TM binds actin very weakly when compared to native TM due only to a lack of N-terminal acetylation (Urbancikova and Hitchcock-DeGregori. 1994. *J. Biol. Chem.* 269:24310), while unacetylated, recombinant smooth TM binds well to actin. Cho and Hitchcock-DeGregori (1991. *PNAS* 88:10153) have used this property to show that the alternatively spliced ninth exon is primarily responsible for the functional differences between unacetylated, recombinant smooth and striated TMs. The smooth ninth exon is required for strong actin binding without Tn, while the striated ninth exon is required for Tn with Ca^{2+} to enhance the binding of TM to actin.

In this study, we examined the functional domains within the ninth exon responsible for (1) TM binding to actin without Tn, (2) Tn to promote TM binding to actin, and (3) regulation of the actomyosin Mg^{2+} ATPase. We have analyzed striated muscle α -TM and a series of unacetylated, recombinant TM ninth exon chimeras and truncation mutants expressed in *E. coli*. The ninth exon chimeras contain the first 18 residues of the smooth or striated ninth exon with the last nine residues of the other exon 9; the truncation mutants lack the last nine residues of the ninth exon. Binding of TM to actin was measured by cosedimentation and analyzed using SDS-PAGE. Our results show that the last nine residues of the smooth ninth exon are required for actin binding without Tn. With Tn and Ca^{2+} , the entire striated ninth exon is necessary for Tn to promote actin binding. With Tn in the absence of Ca^{2+} , either the first 18 or the last nine residues of the striated ninth exon are sufficient for Tn to promote TM binding to actin. All ninth exon variants which bind actin with Tn without Ca^{2+} regulate the actomyosin ATPase, consistent with Cho and Hitchcock-DeGregori (1991. *PNAS* 88:10153). Our results suggest that for both actin binding and regulation with Tn in the absence of Ca^{2+} , either the first 18 residues of the striated ninth exon are sufficient or the entire smooth exon 9 is required. (Supported by NIH, AHA-NJ)

ISOLATION AND CHARACTERIZATION OF MYOSIN I FROM
PORCINE AORTA MEDIA SMOOTH MUSCLE

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There are increasing number of myosin family in recent years. Classic type of myosin is a motor protein of muscle cell. It is so-called myosin II that has two head with long tail where the intermolecular association site exists for the thick filament formation. The motor function localized in the head portion, S1. A new type of myosin family had been discovered in lower eukaryotes and avian intestinal brush border. The new family unable to form thick filament and has a single head with short tail. In non-muscle cells co-existence of myosin I and II has been shown. A clear difference in their intracellular localizations have shown to suggest distinct roles for each type of myosin. Little is known about the presence of myosin I in vertebrate smooth muscle cells .

In this study myosin I has been isolated and identified from porcine aorta media smooth muscle. The protein consists of 110kDa heavy chain with 17kDa light chain that is identified as calmodulin. Each heavy chain can bind two moles of calmodulin. This protein shows a reversible F-actin binding depending upon the absence or presence of ATP. A capability of phospholipid binding has also been determined. Maximum velocity of the actin activated Mg^{2+} ATPase reaction was 151 nmol/min/mg at an apparent affinity constant against actin of 11 μ M in the absence of calcium. The chymotryptic fragments seemed to be quite similar to those of brush border myosin I. From these criteria the isolated protein is identified as myosin I from aorta media smooth muscle. During this work myosin I from urinary bladder smooth muscle was isolated by Chacko et al (1994). The existence of myosin I with abundant myosin II is quite interesting to find the physiological roles in smooth muscle cell.

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HS1, AN SH3 PROTEIN, REQUIRES ONLY A SINGLE COPY OF
37-AMINO ACID REPEAT FOR ITS BINDING TO ACTIN FILAMENT.

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Human HS1 (Hematopoiesis Specific), Cortactin/EMS1 (Chromosome Eleven, Mammary and Squamous Carcinomas-Associated) and a single-headed myosin (Myosin IB) each contains a single SH3 domain at the C-terminus. HS1 and EMS1 SH3 domains share 80% sequence identity and both share 50% sequence identity with myosin IB SH3 domain. Furthermore, EMS1 and myosin IB each contains a single ATP-insensitive F-actin binding domain next to the SH3 domain. It has been shown previously that the actin-binding site of EMS1 is located within an N-terminal domain (residues 45 to 283). Interestingly, this N-terminal domain of EMS1 contains at least five consecutive copies of an internal, tandem 37-amino acid repeat, whereas the corresponding domain of HS1 contains only three consecutive copies of a similar 37-amino acid repeat.

In this paper we demonstrate for the first time that (i) full length HS1 of 487 amino acids indeed binds F-actin, and that (ii) any single copy of the HS1 37-amino acid repeats (residues 82-118, 119-155 or 156-192) is sufficient for the actin-binding, and multiple copies of this unique repeat progressively increase the apparent affinity for F-actin.

Like tumor suppressor p53, HS1 is required for apoptosis of B-lymphocytes. Thus, we are currently examining the possible anti-oncogenicity of HS1 by over-expressing HS1 gene in v-Ha-Ras-transformants.

In addition, using a series of Dictyostelium myosin IB N- and C-terminal deletion mutants, we have found that the minimal ATP-insensitive actin-binding fragment of the myosin IB is an 111-amino acid stretch (residues 948-1058) within its Gly-Pro-Gln-rich (GPQ) domain next to the C-terminal SH3 domain.

INTERACTION OF THE CYTOPLASMIC DOMAIN OF ICAM-2 WITH α -ACTININ

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ICAM-2 is an integral membrane protein expressed on lymphocytes, monocytes and endothelial cells. It functions as a ligand for LFA-1 (CD11a/18) and mediates cell-to-cell contacts. To study the intracellular interactions of ICAM-2 we synthesized a peptide encompassing cytoplasmic amino acids 231-254. In affinity chromatography several proteins of placental lysate bound to this peptide. One of them reacted with α -actinin antibodies in immunoblots. Purified, ¹²⁵I-labeled α -actinin also bound to the peptide immobilized on Sepharose beads. This binding could be competed with unlabeled α -actinin and occurred also in high salt concentration and in the presence of EDTA, whereas denaturing of the protein abolished binding. To further investigate the site of interaction we synthesized overlapping octapeptides covering the entire ICAM-2 cytoplasmic domain. The peptide representing amino acids 241-248 bound α -actinin most avidly and was able to compete with the longer cytoplasmic peptide for α -actinin binding. To determine the interaction site of α -actinin we used ¹²⁵I-labeled α -actinin-GST-fusion protein constructs in binding studies. None of the four individual spectrin-like repeat domains of α -actinin (R1-R4) bound separately to the ICAM-2 cytoplasmic peptide. The fusion protein which contained the entire C-terminal half of α -actinin (repeat domains 3 and 4 and calcium-binding EF-hand like domain) mediated highest binding. However, the N-terminal construct and the fusion protein containing all four repeats (R1-R4) also bound to ICAM-2, indicating that several regions of α -actinin are involved in interaction. These results, together with previous studies demonstrating an interaction between α -actinin and ICAM-1, β 1-integrins and CD18 emphasize the role of α -actinin as a linker between cell surface adhesion molecules and actin-containing cytoskeleton.

INTERACTIONS OF THE DOMAINS OF RADIXIN *IN VIVO* AND *IN VITRO*. Michael Henry, Charo Gonzalez Agosti, Adam Grancell, Margaret Magendantz and Frank Solomon. Dept. of Biology and Center for Cancer Research, M.I.T., Cambridge MA 02139.

The highly related ERM proteins - ezrin, radixin, and moesin - are enriched in cortical cytoskeletal domains. These molecules may provide a structural link between the plasma membrane and underlying cytoskeleton. To identify the interactions of these molecules, we have studied properties of full-length and domains of radixin *in vivo* and *in vitro*. By stably expressing epitope-tagged versions of these polypeptides in cultured animal cells, we could determine how the domains contribute to radixin's localization. In stably transfected NIH-3T3 cells, the full-length molecule localizes to cortical structures - filopodia, lamellipodia, microvilli, and cleavage furrows - as do endogenous radixin and moesin. The carboxy-terminal fragment localizes in a pattern similar, but not identical, to endogenous radixin; it is present in most of the appropriate cortical structures, but conspicuously absent from cleavage furrows. In these experiments, the amino-terminus does not show discrete localization. In transfectants expressing the full-length tagged radixin at levels comparable to the endogenous molecule, or the carboxy-terminal fragment at much lower levels, endogenous moesin immunoreactivity is markedly reduced in cortical structures. Otherwise, stable expression of full-length or truncated form of radixin causes no obvious cellular phenotype. At substantially higher levels of expression in transient transfections, the amino-terminus does localize to the appropriate cortical structures, including cleavage furrows, as does the full-length molecule. However, the same high level expression of the carboxy-terminus results in a significant re-organization of microfilaments, disruption of cell morphology, and inhibition of cytokinesis. Taken together, the results indicate that targeting to cortical structures depends on the cooperation of distinct qualitative and quantitative determinants residing in both the amino- and carboxy-terminus of radixin. They also suggest a model for the molecular organization of radixin in which the amino-terminus regulates *in cis* the interactions of the carboxy-terminus. The apparent displacement from cortical structures of moesin by exogenous radixin suggests they compete for limiting cellular factor(s) necessary for normal localization. *In vitro* binding experiments provide preliminary evidence for such binding partners, and are consistent with the notion that the interactions of the individual domains are modified in the full-length molecule.

THE USE OF FILTER CRYO-ELECTRON MICROSCOPY TO REVEAL DETAILS OF THE ACTOMYOSIN INTERACTION

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We are using the method of filter cryo-electron microscopy to analyse the actomyosin rigor complex. Vitreous ice preserves decorated actin for electron microscopy in its native form but the thick protecting layer of ice gives rise to considerable inelastic scattering and to a high background. Because this adds to the general noise, it limits the resolution obtainable by image processing to worse than 20Å. Fortunately, an energy filter allows the inelastically scattered electrons to be eliminated from the image leading to much clearer pictures (1) at lower dose and also allows the possibility of correcting accurately for the contrast transfer function (2). Using the Zeiss EM912 Omega it is now in principle possible to obtain images in ice with an intrinsic resolution of about 0.8nm.

Actin and myosin have been assembled into a model for the rigor complex by building the component molecules into electron density maps derived from cryo-electron microscopy (3,4). On the basis of these results Rayment *et al* suggested that the well-developed cleft in the 50K segment of myosin S1 might shut on binding to actin thereby providing a link between the actin binding site and the nucleotide binding site on myosin. This idea could be central to an understanding of muscle contraction and checking this hypothesis is a one aim of the present work. We are examining the preservation of detailed structural order during freezing and are correlating and merging images to obtain the necessary resolution. We have developed a new numerical method for 3D reconstruction which allows higher resolution. Our present results achieve a resolution of better than 20Å. These allow new detail to be discerned which indeed leads to more accurate positioning of the actin filament and the myosin cross bridge in the em 3D reconstruction. This in turn allows the actomyosin interface to be defined with better precision. While the general nature of the interaction is as reported by Rayment *et al* we begin to see small modifications to the actin filament structure produced by the docking.

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ACTIN CAPPING PROTEIN FUNCTION IS ESSENTIAL IN *DROSOPHILA*.

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Actin Capping protein is an heterodimeric protein thought to regulate actin filament polymerization by binding to and preventing addition of subunits at the fast growing end. It may also be important for anchoring actin filaments at the Z-disc of skeletal muscle. Our experiments in the fruit fly *Drosophila melanogaster* focus on obtaining evidence for these functions through localization studies and mutational analysis. As the first step in this analysis we cloned the genes encoding the α and β subunits of Capping protein. The β subunit of *Drosophila* is remarkably similar to those of other organisms, particularly the chicken, to which it is 83% identical. Expression of *Drosophila* Capping protein in yeast mutant strains lacking endogenous Capping protein rescues the mutant phenotype. Also, our immunofluorescence studies demonstrate that Capping protein is present in the Z-discs of adult muscle, as previously shown for chicken muscle. These findings suggest that *Drosophila* Capping protein is functionally analogous to Capping proteins characterized in other systems.

We have mapped the gene encoding the β subunit to section 22A on the polytene chromosome map. EMS screens in this region have defined 12 lethal complementation groups, one of which is rescued to adulthood by a β subunit transgene. Since loss of Capping protein function is lethal, this indicates that Capping protein performs an essential function or functions during the *Drosophila* life cycle. We and others have generated 9 lethal alleles of this gene, and we are currently analysing their phenotypes in more detail.

We are using antibodies against Capping protein to examine its distribution during oogenesis, embryonic development and muscle development in wild type flies. We are also studying Capping protein localization in muscle from flies with mutations in other muscle components. These studies, in combination with our genetic analysis, should provide information on the function of Capping protein *in vivo* in both muscle and non-muscle cells.

MICROTUBULE ASSOCIATED PROTEINS FROM INTERPHASE AND MITOTIC-LIKE SEA URCHIN EGG EXTRACTS

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Cytoplasmic extracts from sea urchin eggs (*S. purpuratus* or *L. pictus*) show microtubule (MT) assembly dynamics typical of interphase cells, while addition of the phosphatase inhibitor okadaic acid (OA) results in mitotic-like MT assembly (J. Cell Biol. 119:1271). To begin to understand cell-cycle regulation of MT assembly, we examined the high affinity MT associated proteins (MAPs) co-pelleting with taxol-MTs from interphase and OA treated extracts. These MAPs were further purified by salt-extraction and rebinding to purified porcine brain taxol-MTs. Consistent with previous studies, we find MAPs of 77, 90, 100, 110, 120, 205, and 235kD from interphase extracts. OA extracts contained the same MAPs but consistently showed higher concentrations of the 110 and 235kD polypeptides. Examination of ^{32}P incorporation showed that interphase MAPs of Mr 77, 90, 205, and 235kD are weakly phosphorylated. OA treatment results in a much higher level of phosphorylation of all MAPs. Using DIC video-microscopy to visualize individual MTs, we find that addition of 1 mg/ml interphase or OA MAPs to 12 μM tubulin caused a modest (< 2 fold) increase in MT elongation velocity at both MT ends. The other parameters of dynamic instability were not significantly altered. Our results demonstrate that the composition of high affinity MAPs does not change upon addition of OA. Furthermore, it is unlikely that these MAPs are responsible for the changes in MT assembly dynamics between interphase and mitosis, and our results suggest that proteins which bind to MTs with lower affinity may be the major modulators of MT dynamic instability *in vivo*.

**MICROTUBULE ASSOCIATED PROTEIN (MAP) 1A AND 1B:
PURIFICATION, INTERACTION WITH MICROTUBULES AND
MICROFILAMENTS, AND KINETICS OF TUBULIN POLYMERISATION.**

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High molecular weight microtubule associated proteins (HMW-MAPs) isolated from brain promote microtubule assembly, alter microtubule dynamics, and crosslink microtubules with other cytoplasmic structures. Modulation of these activities implies an important role for these proteins in the organisation of the neuritic cytoskeleton. Of the different HMW-MAPs, MAP1A (350kDa), MAP1B (330kDa), and MAP2 (280kDa), MAP2 is perhaps the best characterised both in terms of its interaction with microtubules and with other cytoskeletal structures. Unlike MAP2, which can be easily purified, MAP1A and MAP1B proteins are difficult to purify and consequently have not been biochemically characterised.

We have developed purification protocols for MAP1A and MAP1B which allow the isolation of milligram quantities of these proteins. The ability of purified MAP1A and MAP1B to bind to microtubules was studied and shows that the stoichiometry of binding to microtubules was 1 mole of MAP1A:16 tubulin dimers and 1 mole of MAP1B:10 moles tubulin dimers. Both MAP1A and MAP1B were able to induce nucleation and elongation of pure tubulin into microtubules. Kinetic analysis of MAP1A- and MAP1B-promoted microtubule assembly yielded association rate constants of $40 \times 10^6 \text{ M}^{-1} \text{ S}^{-1}$ and $200 \times 10^6 \text{ M}^{-1} \text{ S}^{-1}$ respectively. Both constants were significantly higher when compared with that for MAP2 ($20 \times 10^6 \text{ M}^{-1} \text{ S}^{-1}$). Electron microscopy of MAP1A-, MAP1B-, and MAP2 microtubules also demonstrated interesting differences in microtubule length and shape. MAP1B-microtubules were longer and "bendy" while MAP1A-microtubules were short and "stiff rod-like" structures. MAP2-microtubules demonstrated an intermediate length and existed in both "bendy" and "straight" forms. Co-incubation studies showed that MAP2 did not displace microtubule-bound MAP1A and both MAPs were able to colocalise on microtubules. Similar co-incubation experiments, however, showed that MAP2 totally displaced microtubule-bound MAP1B.

The ability to bind to microfilaments was also studied. MAP1A, like MAP2, was able to bind to G- and F-actin and crosslinked microfilaments. By contrast, MAP1B neither bound to G-actin nor F-actin nor was able to co-sediment with actin filaments.

The differences and similarities between the interactions of MAP1A, MAP1B, and MAP2 coupled with their spatial and temporal distribution will be discussed in the context of developmental regulation of the neuritic cytoskeleton.

IDENTIFICATION OF DOMAINS THAT SPECIFY CELL-CYCLE DEPENDENT TARGETING OF CENP-F TO THE KINETOCHORE, AND THE ROLE OF PROTEIN PHOSPHORYLATION IN LOCALIZATION

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CENP-F is a recently identified kinetochore phosphoprotein whose distribution varies during the cell cycle. CENP-F is localized to the nucleus during interphase and assembles onto kinetochores as chromosomes condense during prophase. Its presence at kinetochores is maintained throughout metaphase, and it is redistributed to the spindle midzone at anaphase. To investigate the mechanism that temporally regulates the assembly of CENP-F to kinetochores, studies have been initiated to determine the nuclear- and kinetochore-localization domains of CENP-F. DNA constructs consisting of overlapping fragments of the CENP-F gene fused in-frame to an epitope tag were transfected into HeLa cells and examined by indirect immunofluorescence for their localization at different stages of the cell cycle. The COOH-terminal portion of the CENP-F protein was found to direct the fusion protein to the nucleus at interphase and to kinetochores at prophase and metaphase. To determine the role of protein phosphorylation in the localization of the COOH-terminal portion of CENP-F, in vitro phosphorylation studies have also been initiated. The COOH-terminal portion of CENP-F which is important for nuclear and kinetochore localization was phosphorylated in vitro by cdk2/cyclin A and cdc2/cyclin B complexes which had been isolated from baculovirus-infected cells expressing the kinases and cyclins. Phosphoamino acid analysis of the phosphorylated CENP-F protein revealed exclusive phosphorylation on serine residues. These results indicate that cell-cycle kinases may play a role in the regulation of the temporal localization and function of CENP-F.

PKC BINDING PROTEINS

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Protein kinase C is a family of phospholipid-dependent kinases important for regulating cell growth and differentiation. Most cells express more than one type of PKC indicating that individual isozymes have unique functions. However, phosphorylation studies have demonstrated little difference in substrate specificity among the PKCs in *in vitro* assays. Thus, it seems likely that differential subcellular localization plays a role in limiting the accessibility of individual PKCs to specific substrates *in vivo*. We have determined that PKCs α , δ , ϵ and ζ are differentially localized in REF52 fibroblasts by immunofluorescence with isozyme-specific antibodies. PKC α colocalizes with talin in focal contacts of REF52 cells indicating that PKC α in particular is targeted to these discrete locations. We have used an overlay assay for interaction cloning of proteins that bind PKC and may be responsible for targeting individual PKCs to specific subcellular addresses. Of the more than 12 binding proteins identified to date, all are PKC substrates and most appear to be associated with cytoskeletal structures. The list of binding proteins/substrates identified so far includes vinculin, talin, MARCKS, MARCKS-related protein, desmoyokin, annexins I and II, new members of the adducin and kinesin light chain families and several unique sequences. Alignments of the binding proteins have not yet identified a consensus sequence for PKC binding. Domain mapping studies are in progress to determine PKC contact sites within the binding proteins. In contrast to normal cells, PKC α does not colocalize with substrate attachment points in transformed REF52 cells. Lack of targeting correlates with decreased expression of several PKC binding proteins. We propose that changes in the localization of PKCs and expression of downstream targets contributes to the disordered cell signaling and growth regulation characteristic of transformed cells.

GENOMIC STRUCTURE OF HUMAN MICROTUBULE-ASSOCIATED PROTEIN-2 (MAP-2) AND IDENTIFICATION OF 6 NOVEL EXONS.

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Analysis of overlapping clones from several human genomic libraries and PCR analysis of human DNA has determined the genomic organization of human microtubule-associated protein-2 (MAP-2). The gene spans a region of chromosome 2 which is approximately 150 kilobases. The exons vary in size from 62 bp to greater than 3 kb and are separated by introns ranging in size from 170 bp to over 10 kb. Six novel exons, exons 1, 2, 3, 4, 8 and 13, have been identified and studies indicate that these MAP-2 exons are all transcribed. Exons 1, 2 and 3 encode alternate 5' untranslated regions spliced to exon 4, which contains an additional 141 bp of 5' untranslated region utilized by all MAP-2 transcripts. The remaining -29 bp of the 5' UTR and the initiation start site for translation are located in the fifth exon. The RII binding domain of cAMP-dependent protein kinase is encoded within exons 5 and 6. The large region of HMW MAP-2 which is spliced out of MAP-2c transcripts is contained within exons 9, 10 and 11. With the exception of the fourth repeat of the microtubule-binding domain which is within a single exon, the first, second and third repeats are either split between two exons or are within exons containing adjacent MAP-2 coding sequences. Exons 8 and 13 are novel exons which are transcribed in human adult brain, fetal spinal cord and the human neuroblastoma cell line MSN. The 3.3 kb 3' untranslated region is within a single exon which also contains 213 bp of coding sequence and the translational stop site.

A cDNA CLONE ENCODING A PROTEIN SIMILAR
TO MOESIN, EZRIN AND RADIXIN IN A
MEALYBUG, *PLANOCOCCUS LILACINUS*

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Two features of the mealybug genetic system are of unusual interest: of the two haploid sets of chromosomes, that from the father is inactive in sons but not in daughters. The chromosomes are holocentric, i.e., centromeric activity is distributed all along the length of the chromosome. During a search for genes showing sex-specific and developmental stage-specific expression, a cDNA clone (P3, 2.1 kb) showing close similarity to moesin, ezrin and radixin proteins was identified. P3, which is expressed at all stages of development, hybridizes to two distinct transcripts in embryos, males and females. Southern analysis indicates that the organization and copy number of this gene is not different in the two sexes. *In situ* hybridization to RNA in whole-mount embryos shows that P3 transcripts are uniformly distributed throughout the embryo. The amino acid sequence derived from the largest ORF of this clone shows 70-75% similarity to the amino terminal portion of a class of mammalian cytoskeleton-associated proteins which includes moesin, radixin and ezrin. Its similarity to merlin (NF2 tumor suppressor protein) is about 60%.

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CHROMOSOME ARCHITECTURE IN CULTIVATED PEARL MILLET

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In pearl millet, *Pennisetum americanum* (L.) Leeke or *P. typhoides* (Burm.) S.&H., a systematic study was carried out to accumulate large number of cells at prophase and metaphase stages for the establishment of its karyotype. A combination of solutions of different concentrations and durations of colchicine, α -bromonephthalene and p-dichlorobenzene as pretreatment agents was used. In one set of experiments, the roots were treated with pre-cooled distilled water at 2-4°C and distilled water at 25±1°C for different durations. We found that with pretreatment of roots, metaphase index (Me.I.) was in the range of 0.50 and 0.75 per cent. With the increase in concentration and duration of colchicine (0.01 to 0.1 per cent; 5 to 215 minutes), a gradual decrease in Me.I. was displayed. Highest mitotic index (Mi.I.) of 6.0 per cent was observed when roots were treated with pre-cooled distilled water at 2-4°C for 60 minutes. Whereas, Mi.I. of 4.0 was shown in case of treatment of roots with water at 25±1°C for 10 minutes. With Me.I. were 1.21 and 1.23 per cent, respectively. Overall effect of water treatment was terms of optimal shortening of chromosome prometaphase stage. This facilitated identification of major as well as minor bands for the of specific pearl millet chromosomes.

**DISRUPTION OF THE HEPATOCYTIC CYTOSKELETON IN
ALCOHOLIC LIVER DISEASE AND RELATED ANIMAL MODELS**

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Alcoholic hepatitis in humans as well as chronic Griseofulvin (GF) or 1,4-diethoxycarbonyl-2,4-dihydrocollidine (DDC) intoxication in mice result in a disruption of the hepatocytic keratin filaments and B-type nuclear lamins. These alterations are accompanied by the appearance of keratin-containing cytoplasmic inclusions termed Mallory bodies (MBs) in hepatocytes. MBs share some morphological, biochemical and immunological properties with cytoplasmic protein inclusions in neurons (Neurofibrillary tangles; NFT's) of brains of patients with Alzheimer's disease. Recently, we detected in MB's abnormally phosphorylated tau, which is also a major constituent of NFT's. We show here, that different tau isoforms are expressed in murine brain and liver. Using RT-PCR, we cloned four different tau sequences, which showed alternative splicing patterns of exon 2, 3, 4 and 10. Chronic GF or DDC intoxication leads to a liver-selective overexpression (up to 30 fold) of tau mRNA. This overexpression of tau is restricted to the major tau isoform, whereas the minor isoforms are almost completely repressed. In contrast to tau, there is a reduction in the expression of tubulin-M α 2 during GF or DDC intoxication. These changes are reversible after recovery from intoxication.

The dissoziation between tau and tubulin expression suggests that hyperphosphorylation counteracts the overexpression of tau so that under these conditions a destabilization of microtubules occurs. This mechanism might be a new explanation for the disturbance of microtubule-dependent processes in the liver, which are observed in alcoholic liver disease.

PEROMONE RECEPTOR-INDUCED MORPHOGENESIS.

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In the yeast *S. cerevisiae*, mating pheromones stimulate a G protein-coupled receptor that induces cells to depart from the usual bud morphogenesis program and to instead form an acute projection that becomes the site of cell fusion during conjugation. Genetic analysis indicates that the cytoplasmic C terminus of the α -factor pheromone receptor promotes projection formation by a mechanism that is distinct from G protein activation. We also identified a pheromone-stimulated gene, *AFR1*, that acts in this pathway together with the receptor. The *AFR1* protein does not appear to act directly on actin since immunolocalization studies detected *AFR1* at the base of projections whereas the majority of actin was detected at the leading edge of the projections. However, *AFR1* was found to interact with the *CDC12* protein in the two-hybrid system and the two proteins localize to similar sites in vivo. Work from the Pringle lab indicates that *CDC12* belongs to a family of putative filament-forming proteins that are required for normal bud morphogenesis. The function of *CDC12* in projection formation has not been studied extensively, but *cdc12^{ts}* mutants are also defective in projection formation. Therefore, the significance of the interaction between *AFR1* and *CDC12* was tested by examining *AFR1* mutants. Mutations in *AFR1* that destroy the interaction with *CDC12* caused altered localization of the *AFR1* protein and a defect in projection formation. The defect in projection formation could be partially suppressed by overproduction of the mutant *AFR1* proteins. Altogether, these results suggest that interaction with *CDC12* is important for the proper localization of *AFR1*, but is not necessary for *AFR1* to have effects on morphogenesis. Experiments are now underway to identify the other components that act in this α -factor receptor-induced morphogenesis pathway.

Background References:

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AXONAL ANKYRINS: GIANT ANKYRIN ISOFORMS INVOLVED IN DIFFERENTIATION OF THE AXONAL PLASMA MEMBRANE.

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A number of ankyrin-binding proteins have been identified in the axonal plasma membrane. Members of the neurofascin/L1 family of cell adhesion molecules, the Na/K-ATPase, the Na/Ca exchanger and the voltage-dependent sodium channel have all been shown to bind to the peripheral protein ankyrin, which is believed to interconnect integral membrane proteins with the spectrin-based membrane skeleton. We have discovered a family of human "giant" ankyrin isoforms, uniquely targeted to the axonal membrane. Members of this family are characterized by an alternatively spliced domain that introduces a long unstructured tail domain into the ankyrin molecule. This domain may be up to 200nm in length, allowing the isoform to have interactions with both the plasma membrane and components of the axonal cytoplasm such as neurofilaments and microtubules. The first of these isoforms to be characterized was a 440kDa alternatively spliced isoform of the ankyrin β gene (Chan *et al*, J. Cell. Biol. **123**, pp1463-1473, 1993). This isoform is selectively localized to unmyelinated and premyelinated axons from where it is downregulated during myelination.

We now describe the identification and characterization of a second "giant" ankyrin isoform produced by alternative splicing of a novel ankyrin gene, ankyrin γ . This gene is expressed in many tissues with multiple isoforms being produced by alternative splicing. Some of these isoforms lack the membrane-binding domain of ankyrin suggesting that they may segregate to cellular compartments other than the plasma membrane; however, the two largest spliceforms of 480kDa and 270kDa are observed only in neural tissue. The ankyrin γ 480kDa is 71% identical to the ankyrin β 440kDa and has a sequence that suggests a long unstructured "tail" region connected to globular head domains associated with spectrin and membrane binding. In addition, both the 480kDa and 270kDa isoforms also have a novel serine-rich domain that may be a candidate for post-translational modifications such as O-linked glycosylation. Antibodies against this serine-rich domain show that these large isoforms are also observed only in axons, where they are uniquely localized at the axon initial segment and node of Ranvier of myelinated axons. These are the first unique cytoplasmic components localized to these highly differentiated areas of the axonal plasma membrane. They provide an important tool to understanding how these physiologically significant membrane domains are established. Many of the ankyrin binding proteins mentioned earlier are also localized to the axon initial segment and node of Ranvier. For instance, highly localized concentrations of the voltage-dependent sodium channel at the initial segment and node are critical to the initiation and propagation of the saltatory action potential.

Preliminary developmental studies show that localization of ankyrin γ spliceforms at the axon initial segment occurs early in axonal development and that this protein may represent an early marker for axonal polarity. Clustering of ankyrin γ at presumed sites of nodal formation is also an early event in myelination, suggesting that the 480kDa and 270kDa have a role to play in establishing the node of Ranvier.

HEF1, A PUTATIVE ADAPTOR PROTEIN INVOLVED IN COORDINATING GROWTH CONTROL SIGNALS AND CYTOSKELETAL CHANGES

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The pseudohyphal budding pathway of the yeast *S. cerevisiae* follows a genetically programmed pattern of cell division, set asymmetry, and morphology, which can be regulated by external signals. Furthermore, many mammalian genes whose products are known to interact with both the cell cytoskeleton as well as mitogenic growth signaling pathways (Dbl, Vav, Bcr, Ect-2, KRev-1, and Tiam-1) have functionally conserved homologs in the yeast budding pathway that control yeast morphology and progression through the cell cycle. We have used the enhancement of pseudohyphal growth by clones expressed from a HeLa cDNA library as a novel screen to identify human genes involved in the process of coordinating cell cycle and cell morphological changes.

HEF1 (Human Enhancer of Filamentous growth-1) was isolated as a 900 bp cDNA encoding a novel ORF that caused constitutive formation of pseudohyphae in diploid *S. cerevisiae*. Using this clone as a probe, we have isolated a 3.4 kb cDNA containing a much larger ORF which may correspond to an alternatively spliced transcript. The isolated HEF1 clone contains a predicted SH3 domain, multiple SH2 binding motifs including those thought to be the consensus sequences for Crk binding, and a novel third domain corresponding to an exon containing the original shorter ORF isolated in the pseudohyphal screen. Overall, the predicted protein is quite similar to the newly described Crk-binding protein p130cas, which becomes hyperphosphorylated during Crk-mediated cellular transformation, and is thought to be an adaptor protein responsible for assembling signals from SH2-domain containing oncoproteins including Crk, Src, Abl, and Arg. In contrast to the ubiquitous distribution of p130Cas, Northern analysis of human tissues reveals two transcripts of 3.2 and 5.0 kb which were highest in kidney, lung, and placenta, moderate in heart, liver and skeletal muscle, and lowest in brain and pancreas. Two other transcripts were detected one of 8.0 kb in kidney and lung and one of 1.2kb in liver. A polyclonal antiserum specific for HEF1 detects multiple size proteins (84, 48, and 40kD) in HeLa, NIH3T3, MDCK, HEK293, and PC12 cells, which may correspond to the multiple transcripts detected by Northern analysis. To test whether HEF1 is hyperphosphorylated like p130cas, lysates from v-abl and v-crk transformed cells were immunoprecipitated with the anti-HEF1 antiserum and detected by Western analysis with anti-phosphotyrosine antibodies. HEF1 was hyperphosphorylated in both cell lines. Together, these results suggest that HEF1 may be an adaptor protein which links cell division signals with those involved in remodeling the cytoskeleton.

ACTIN BINDING PROTEIN PRESENT IN NEURONAL NUCLEI

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A monoclonal antibody, 54A1, was produced against a crude preparation of actin associated proteins. 54A1 reacted with an actin binding protein, molecular weight about 50 Kd. The peptide was present in most human and bovine tissue as well as various cultured cells such as Hela, MDCK, and HepG2.

By immunofluorescence microscopy using cultured cells, the target protein was present in the cytoplasm as fine dots which were associated with "stress fibers". They were present diffusely along the stress fibers; however, they were more densely distributed at the periphery of the fibers especially where the cytoplasm was protruded. By double immunofluorescence microscopy, they were associated with actin filaments while neither intermediate filaments nor microtubules were associated.

In human tissue samples, they were present in the cytoplasm of most cells, especially at the connective tissue cells. Among the epithelial cells, only certain types were immunostained. However, in the neurons at the central as well as peripheral nervous system, the immunostaining was present not only in the cytoplasm but also in the nuclei. The nuclei were diffusely and strongly immunostained with the only exclusion of nucleoli, while the neuronal cytoplasm was similarly immunostained as other cell types.

The c-DNA cloning is under progress.

THE REGULATION OF KERATOCYTE LOCOMOTION

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Cell movement along a substratum involves the coordination of several events; adhesion and extension of the leading edge; retraction and detachment of the trailing edge. Fish epidermal keratocytes exhibit a relatively simple mode of locomotion in which cell shape, speed and direction of movement remain almost constant. This is due to the high degree of coordination between extending and retracting edges. Recent work indicates that this depends on the ratio between cell - substratum adhesion strength and lamellar contractility along the cell margin. At the leading edge, where large cell - substratum contacts exist and lamellar contractility is low, actin polymerization occurs most rapidly. It also occurs perpendicular to the cell margin and in a graded manner so that cell shape is maintained. However, towards the cell rear, cell - substratum contacts decrease in size but lamellar contractility increases. Where contractility exceeds cell - substratum adhesion strength retraction of the cell margin occurs, inwards, perpendicular to the cell edge. Retraction and extension occur simultaneously so that keratocyte size and shape is maintained.

A key to understanding keratocyte locomotion lies in how cell - substratum adhesion and contractility are regulated and how this is related to the control of actin filament dynamics. The regulation of intracellular calcium concentration $[Ca^{2+}]_i$ is required for cell movement. In some cells an increasing gradient of $[Ca^{2+}]_i$ from front to rear of the cell is thought to favor extension at the front and retraction at the rear of the cell. To investigate the role of $[Ca^{2+}]_i$ in keratocyte locomotion we have photoactivated caged IP3 within specific regions of the keratocyte lamella, and observed the resulting changes in $[Ca^{2+}]_i$ and associated changes in cell morphology. These results will be presented and discussed in relation to keratocyte locomotion.

IN VITRO ANALYSIS OF CORTICAL ACTIN ASSEMBLY DURING YEAST POLARIZED CELL GROWTH

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We have established an *in vitro* assay for the assembly of cortical actin cytoskeleton that is essential for the polarized growth of budding yeast cells. After permeabilization of yeast cells by a novel procedure designed to maintain the spatial organization of cellular constituents, exogenously added fluorescently-labeled actin monomers assemble into distinct structures similar to the cortical actin patches formed *in vivo*. The pattern of the cortical actin assembly *in vitro* also reflects the *in vivo* actin distribution through out the cell cycle. Actin polymerization in the bud of the permeabilized cells requires a nucleation activity provided by protein factors distinct from the barbed ends of endogenous actin filaments. This nucleation activity is lost in cells that lack Sla1 or sla2, two proteins previously implicated in cortical actin cytoskeleton function. To biochemically define the nucleation activity, we have established conditions under which a loss of the activity from the bud due to high salt treatment can be complemented *in vitro* using a cytoplasmic extract. We are now in the process of fractionating the extract using complementation as an assay.

We have also used the permeabilized cell assay to investigate the role of the small GTP-binding proteins in regulating cytoskeletal assembly. Actin incorporation in the bud of the permeabilized cells is abolished by mutations in *CDC42* and *CDC24*, encoding a Rho-like GTP-binding protein and its nucleotide exchange factor, respectively, both required for bud formation. Furthermore, the nucleation activity in the bud can be stimulated *in vitro* by a constitutively active Cdc42 protein. These results suggest that Cdc42 may play a direct role in regulating actin assembly during polarized cell growth.

ROLE OF THE CYTOSKELETON IN ACTIVATION OF LFA-1

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LFA-1 is a cell adhesion molecule that belongs to the CD11/CD18 family of integrins. LFA-1 is only expressed on leukocytes. In search of elements that may affect the activation of LFA-1, we have transfected the cDNA of the α and β chain of LFA-1 into two non-leukocytic cells: murine fibroblast cells (L cells) and human erythroleukemic K562 cells. To determine the activation status of LFA-1 on these cells, adhesion assays of these transfectants were performed to the three ligands of LFA-1: ICAM-1, -2 and -3. In contrast to normal leukocytes, LFA-1 expressed on L cells is already active, although a further increase in adhesion to ICAM-1 can be achieved upon addition of different stimuli (PMA, Mn^{2+} and activating anti- β_2 antibodies).

When LFA-1 was expressed in K562 cells, PMA was incapable to induce LFA-1 mediated adhesion. In contrast, the activating anti- β_2 antibodies induced LFA-1 dependent adhesion of the K562 transfectants, indicating that a normal intracellular signaling route necessary for LFA-1 activation is absent.

Coupling and uncoupling of integrins to and from the cytoskeleton may be important in the regulation of integrin-ligand interaction. We have studied the distribution of LFA-1 and actin and α -actinin co-localisation on these different LFA-1 transfectants. Preliminary data suggest that differences in cytoskeletal organisation in the cell may account for differences in the activity of the LFA-1 integrin.

GENETIC ANALYSIS OF TUBULIN REQUIREMENTS FOR AXONEME ASSEMBLY IN *DROSOPHILA MELANOGASTER*

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In the *Drosophila* male germ line, several functionally distinct microtubule arrays are assembled from a single tubulin heterodimer composed of the testis-specific $\beta 2$ -tubulin isoform and the constitutive $\alpha 84B$ -tubulin isoform. We have examined specific aspects of tubulin structure required for assembly of different classes of microtubules. Our results show that assembly of the motile sperm tail axoneme has unique requirements that are not shared by cytoplasmic and spindle microtubules:

[1] We performed isoform swaps to put other native *Drosophila* tubulin isoforms into the male germ line. We have found that heterologous isoforms differentially affect different subsets of microtubules within the axoneme; a heterologous β -tubulin specifically disrupted doublet microtubule morphology, whereas a heterologous α -tubulin disrupted the morphology of singlet axonemal microtubules, but did not affect correct assembly of the doublets.

[2] Some microtubule functions in the male germ line are exquisitely sensitive to changes in the normal tubulin pool level, but axoneme morphology is normally robust to changes in level. However, axoneme assembly becomes sensitive to level if tubulin structure is also compromised. The dose-sensitive phenotype of a recessive male sterile mutation in the $\beta 2$ -tubulin gene has allowed us to formulate a model for regulation of axoneme microtubule morphology as a function of distinct tubulin assembly kinetics for the doublet and singlet microtubules.

[3] We have shown that the $\beta 2$ C-terminus is absolutely required for axoneme assembly, although it is dispensable for assembly and function of the spindle and cytoplasmic microtubules (consistent with the finding that the β -tubulin C-terminus is dispensable in yeast). Examination of a chimeric β -tubulin shows that the $\beta 2$ -specific C-terminal sequences can confer the capability for doublet microtubule assembly and the attachment of the spoke-linker complex on an otherwise axoneme-incompetent heterologous β -tubulin isoform.

[4] Although the role of post-translational modifications remains enigmatic, our results show a correlation between tubulin modification and functional capacity for axoneme assembly for both α - and β -tubulin. We find that in *Drosophila* sperm, both α - and β -tubulin are extensively glutamylated. We observe that α - and β -tubulin structural requirements must both be met in order for glutamylation of either subunit to occur normally. Our results thus suggest that glutamylation may take place only on an axoneme-competent dimer, or on the axoneme itself.

MOLECULAR AND STRUCTURAL ANALYSIS OF TEKTINS IN FLAGELLAR MICROTUBULES. Richard W Linck, Linda A Amos*, Edward H Egelman, Dana Nojima, Jan M Norrander, and Mark A Pimer. Dept Cell Biology & Neuroanatomy, Univ Minnesota, Minneapolis, MN; *MRC Laboratory of Molecular Biology, Cambridge, England.

Tektins are a novel family of proteins associated with ciliary and flagellar microtubules, and evidence suggests that tektin-like proteins are present in other microtubule systems and in species including mammals. Tektins A~53-kD, B~51-kD, and C~47-kD from sea urchin sperm flagellar doublet microtubules are associated with a chemically unique ribbon of 3-4 protofilaments (pf-ribbons), located near the inner junction between the A- and B-tubules. We have taken several approaches to investigate tektin structure and function. **I.** Biochemical crosslinking studies of pf-ribbons have demonstrated that tektins exist as longitudinally continuous filaments in the walls of pf-ribbons and thus of flagellar microtubules, that tektins form polymers composed of a core of tektin A-B heterodimers, and that tektin filaments have an inherent 16 nm axial periodicity that can be accommodated by the tubulin lattice. **II.** Analysis by quantitative electron microscopy concludes that one of the protofilaments of the pf-ribbons and thus of flagellar microtubules is composed of tektin, not tubulin. This conclusion is based on the following: The mass of the unique pf is significantly less than that for tubulin; the pf does not bind kinesin; and the pf has an underlying 48 nm repeat that correlates with tektin's structural features. **III.** The amino acid sequences of all three tektins have been predicted from their cDNAs, and this analysis reveals several structural features: The three tektins are structurally homologous and contain a structural motif not unlike intermediate filament proteins; i.e., the polypeptide chains are predicted to form extended coiled-coil rods, interrupted by short non-helical linkers. The tektin family appears to have arisen from the duplication of a half-length gene. Finally, the length of a coiled-coil is predicted to be ~48 nm long, and in addition each tektin sequence may have the capability for axial repeats of 16 and 24 nm. These combined results indicate that tektins may have a number of important functions in the assembly, structure and function of microtubules.

ANALYSIS OF TUBULIN GENE FUNCTION USING GENE REPLACEMENT IN *TETRAHYMENA*

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In *Tetrahymena thermophila* 17 microtubule systems are produced from a tubulin heterodimer containing a single primary sequence. Thus, microtubule heterogeneity in this organism must be due to either secondary modifications or to MAPs. Both the single α -tubulin gene and the two co-expressed β -tubulin genes (encoding identical proteins) have been cloned. We recently developed methods for mass transformation of *Tetrahymena* in which most transformation events occur by homologous integration. We have created an *in vitro* mutagenized β -tubulin gene (LYS-350 to MET) and isolated a nitrosoguanidine-mutated α -tubulin gene (ALA-65 to THR) that are resistant to microtubule depolymerizing drugs and are hypersensitive to taxol. Cells containing either of these mutant genes can be transformed with the appropriate wildtype tubulin gene and selected for taxol resistance. This system is being used to study the effects of eliminating sites of secondary modification in otherwise wildtype tubulin genes. In *Tetrahymena*, as in most other eukaryotes, α -tubulin is modified by acetylation at LYS-40. In our initial studies we completely replaced all of the expressed α -tubulin genes with a gene containing ARG at residue 40, preventing acetylation. Mutants lacking detectable acetylated α -tubulin are indistinguishable from wildtype both morphologically and physiologically.

Using a Neo gene cassette to interrupt the coding region, each of the two β -tubulin genes was disrupted without any detectable effect on vegetative growth or conjugation. *Tetrahymena* compensate for the loss of one of the β -genes by doubling the amount of mRNA derived from the remaining gene. After deciliation, expression from both β -genes and the α -gene is induced. However, transcription of only one of the two β -genes (BTU1) is induced by treatment with antitubulin drugs. Using mutant tubulin genes with altered drug sensitivities, we have shown that induction of BTU1 and of the α -gene parallel altered drug sensitivity. Thus, in *Tetrahymena*, distinct mechanisms modulate tubulin gene expression depending on whether ciliary or cytoplasmic microtubules are perturbed and the non-ciliary cytoskeleton participates in a signal transduction pathway that regulates specific tubulin gene transcription.

REGULATION OF MICROTUBULE DYNAMICS BY NUCLEOTIDE EXCHANGE INTO ENDS

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The recent discovery that GTP linked to latex beads binds to microtubule ends suggested that nucleotide interactions at this site may play a role in regulating microtubule (MT) dynamics. Evidence for this was sought using DIC microscopy to analyze effects of the free GTP and GDP concentration on the rates of MT elongation and phase transition to rapid shortening (catastrophe, k_c). That nucleotide can dissociate and thereby destabilize the plus end by forming nucleotide-free (apotubulin) subunits, was indicated by the increase in k_c from 0.001 s^{-1} to 0.054 s^{-1} , when the free GTP concentration was reduced from 100 to 0.5 μM , during assembly with 15 μM tubulin-GTP subunits (TuT). That nucleotide can bind to the minus end was indicated by a three-fold decrease in the rate of elongation when the free GDP concentration was increased from 1.6 to 175 μM , during assembly with a mixture of 36 μM TuT and 54 μM TuD. Also, it was found that following dilution of microtubules into tubulin-free buffer the rapid disassembly from the minus end could be stopped (rescued) by the presence of the slowly hydrolyzable GTP analogue GMPCPP (guanylyl-(α,β)-methylene-diphosphonate). Further evidence that nucleotide can bind to both ends of microtubules was provided by the observation that with a mixture of 36 μM TuT and 54 μM Tu-GDP, k_c was increased from 0.0036 to 0.054 s^{-1} at the plus end, and from 0.006 to 0.0006 s^{-1} at the minus end, when the free GDP concentration was increased from 1.6 to 175 μM .

Our evidence for destabilization of microtubules by formation of apotubulin and by nucleotide exchange to form terminal TuD subunits suggests that microtubule dynamics can be regulated in cells by an exchange factor that generates apotubulin subunits, or by a GTPase activating protein that forms TuD subunits at microtubule ends.

MAPPING OF THE TAXOL BINDING SITE ON TUBULIN; A PHOTOAFFINITY LABELING APPROACH.

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Taxol is a compound of considerable interest due to its clinical activity as an antitumor drug in humans, its unusual chemical structure, and its unique mechanism of action. Although it is known that taxol interacts with the tubulin/microtubule system, until recently there has been no definitive information available on the taxol binding site on the microtubule. In the absence of a high resolution crystal structure for the α/β -tubulin heterodimer, photoaffinity labeling studies provide a means to obtain information about the molecular contacts between taxol and its target protein. Two different photoaffinity analogues of taxol, ^3H -3'-(p-azidobenzamido)taxol and ^3H -2-(m-azidobenzoyl)taxol were used. Both photoaffinity analogues photo-incorporate specifically into β -tubulin. The photo-incorporation can be competed by taxol as well as the unlabeled taxol analogues, suggesting a common binding domain. We have reported that 3'-(p-azidobenzamido)taxol photolabels the N-terminal 31 amino acids of β -tubulin (J. Biol. Chem. (1994) 269:3132). Now, we have identified yet another domain of the taxol binding site using the 2-m-azidobenzoyl analogue. Formic acid treatment, which preferentially cleaves Asp-Pro peptide bonds, indicated that the 2-m-azidobenzoyl analogue was bound to a β -tubulin fragment encompassing amino acid residues 32-304. To isolate the photolabeled domain, the ^3H -2-(m-azidobenzoyl)taxol-photolabeled β -tubulin was electroeluted from SDS-PAGE gels, digested with CNBr and passed through a Sephadex G-25 column. The eluate was purified by C-8 reverse phase HPLC and the major radiolabeled peak was treated with trypsin. The radioactive tryptic peptides were fractionated by C-8 reverse phase HPLC. Amino acid sequence analysis of the isolated peptides showed that the 2-m-azidobenzoyl analogue was covalently bound to a peptide containing amino acid residues 217-233 of β -tubulin. This domain and the N-terminal 31 amino acids, although located far apart by primary sequence, must be spatially close enough to bind to taxol in the native tubulin. In addition, these two taxol binding domains are highly conserved during evolution and were also shown to be a part of the colchicine binding site (PNAS (1993) 90:11598), highlighting the functional importance of these domains in drug-tubulin interactions. PCR-based methodology has been developed to sequence each of the six known murine β -tubulin isotype DNAs in the two taxol binding domains of a series of taxol-resistant murine cell lines.

MICROTUBULE AND PROTEIN KINASE-C INTERACTIONS WITH BOVINE MAP-2 MT-BINDING REGION AND 3-/4-REPEAT RAT MAP-2C.

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Neurons achieve and maintain their elaborate ultrastructure at least in part from the ability of the cytomatrix component MAP-2 to alter microtubule (MT) dynamics and structure. Because MAP-2 is extensively phosphorylated *in vivo*, detailed biochemical studies of MAP-2 interactions with other cytoskeletal proteins are severely hampered by the microheterogeneity of MAP-2 isolated directly from brain tissue. Bacterial expression affords one means for defining the interactions of dephospho-MAP-2 with MTs and protein kinases, and we focused on bovine brain MAP-2 microtubule-binding region (*MTBR*) as well as the 3- and 4-repeat forms of rat MAP-2c. We confirmed that the second non-identical sequence repeat *VTSKCGSLKNIRHRPGGG* is the major site for MAP-2 binding to microtubules. Then, by using site-directed mutagenesis to generate CNBR-fragments suitable for time-of-flight mass spectral analysis, we identified S1703 (in the 1st inter-repeat) and S1711 (in the 2nd repeat) as PKC phosphoryl acceptor sites affecting *MTBR*-stimulated tubulin polymerization. Electrostatics are thought to dominate MT-MAP-2 binding interactions, but positive charge is likewise important in directing protein kinases to their target sites. By single and multiple site-specific mutations in this region, we segregated those side-chain positive charges required for MT binding from those serving as PKC recognition sites. Mutants K1708A (preceding S1711 in 2nd repeat) and 1698A/K1699A (preceding S1703 in the 1st inter-repeat) displayed little or no change in *MTBR*-stimulated tubulin polymerization, but essential PKC recognition sites are lost. When viewed in the context of the extreme sequence preservation in all MAP-2 *MTBR*'s to date, our findings suggest that S1703 and S1711 are probably important sites for modulating MAP-2 interactions with MTs in neurons. We have also investigated 3- and 4-repeat forms of rat MAP-2c in terms of (a) MAP-stimulated MT assembly, (b) equilibrium binding to taxol-stimulated MTs, (c) PKC phosphorylation-induced changes in MAP-stimulated tubulin polymerization, and (d) susceptibility to thrombin cleavage. (*Supported by NIH GM-44823*).

ANALYSIS OF MAP 4 FUNCTION IN LIVING CELLS USING GREEN FLUORESCENT PROTEIN (GFP) CHIMERAS

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MAP 4 is a ubiquitous structural microtubule-associated protein thought to play a role in microtubule organization in both interphase and mitotic cells. MAP 4 is composed of three distinct charge domains; an acidic projection domain, a basic region that binds microtubules, and a short acidic C-terminus. Stable over-expression of human MAP 4 in CHO cells has no effect on microtubule organization and does not stabilize microtubules against depolymerizing drugs (Barlow et al, JCB 126:1017-1030). Conversely, transfection with an epitope-tagged construct of the entire basic binding domain of MAP 4 causes extensive microtubule rearrangement (Olson and Olmsted, CSHL Meeting Abstract 1993). We recently analyzed the behavior of various protein domains of MAP 4 in vivo using Green Fluorescent Protein (GFP) chimeras. The fluorescent properties of GFP allow visualization of expressed proteins in live cells and analysis of their effects on microtubule organization. GFP-MAP 4 localizes specifically to microtubules; this is confirmed by the reorganization of the GFP chimera patterns following treatments of cells with anti-microtubule agents and by co-localization of GFP-MAP 4 with microtubules made fluorescent by microinjection of rhodamine-tubulin into cells. Additional analyses reveal distinct contributions of different sub-domains of MAP 4 to microtubule organization and dynamics. The highly conserved PGGG repeats of the binding domain have a very weak affinity for microtubules and are dispensable for microtubule binding, whereas the PSP flanking region appears to function as a binding domain itself. The binding domain alone will stabilize some microtubules against depolymerization with nocodazole. The projection domain shows no microtubule localization, but does modulate association of the binding domain with microtubules. The acidic C-terminus also strongly affects binding interactions, despite constituting less than 1/15 of the total protein. These data show that MAP 4 association with microtubules is modulated by sequences both within the binding domain and the negatively charged flanking regions, and that the GFP chimeras will allow further examination of the effects of this protein and its variants on microtubule dynamics in real time. (Supported by NIH GM22214 and NSF GER 9350145 to JBO and NIH GM36663 to JRMcl.)

ROLE OF MICROTUBULAR SYSTEM IN MORPHOGENESIS AND DYNAMIC ORGANIZATION OF ACTIN IN DISCOID AND POLARIZED EPITHELIAL CELLS

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Organization of morphology and motility of the cells of various types is an important but still poorly understood function of microtubules. This talk summarizes recent data of our group on the roles of microtubules in cultured epitheliocytes. Importance of microtubular control for epithelial morphogeneses in vivo is suggested by the experiments showing that taxol inhibits the formation of systems of branched tubules from cell aggregates in collagen gels. We examined the role of microtubules in transformations of discoid epithelial cells into polarized fibroblast-like cells induced by transfection of mutated N-ras oncogene and by scatter factor ,HGF/SF. Among other methods we used laser trap analysis of cell surface movements (in collaboration with the Rutgers University) and quantitative assessments of pseudopodial activities and of cell shape.

Polarization of pseudopodial activities and dynamic organization of the actin were found to be microtubule-dependent in both systems. Microtubules are essential for the actin organization not only in polarized but also in discoid epitheliocytes. We propose to name this new function of microtubules 'contrapolarization'. Enhancement of actin polymerization in the active edge zone may be the central event in the control of actin dynamics by microtubules in polarized and in contrapolarized discoid cells. Injection of antibodies to heavy chain of kinesin and exposure to Brefeldin A mimic the effects of microtubule-depolymerizing drugs on actin organization suggesting that this control is mediated by microtubule-dependent transport of some organelles regulating actin polymerization.

Reorganizations of oncogene-transfected cells involve two types of cytoskeletal changes :a/diminishment of the total pseudopodial activity due to alterations of actin dynamics; b/ reorganization of microtubular system leading to restriction of pseudopodial activity to one zone of the edge accompanied by considerable enhancement of the local rate of activity in this zone. Interaction of these two components of cytoskeletal changes may be necessary for invasive behavior of neoplastic cells.

A KINESIN-RELATED PROTEIN INVOLVED IN REGULATING MICROTUBULE DYNAMICS DURING MITOTIC SPINDLE FORMATION

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We are investigating the role of kinesin-related proteins (KRPs) in mitosis. To this end, we have used a peptide antibody to a conserved sequence in the motor domain of kinesins to screen a *Xenopus* ovary cDNA expression library. Among the clones isolated were 56 that encoded a protein we named XKCM1 for *Xenopus* Kinesin Central Motor 1. XKCM1 is an 85 kDa protein with an N-terminal globular domain, a central kinesin-like motor domain, and a short carboxy-terminal alpha helical tail. XKCM1 is most homologous to the Kif2 family of KRPs (Aizawa, et al., JCB 119:1287-96). The closest homologue is MCAK, a chinese hamster ovary KRP identified in a screen similar to the one described here (Wordeman and Mitchison, JCB 128: 95-105).

Antibodies raised against the N-terminal globular domain of XKCM1 recognize an abundant 85 kDa protein in *Xenopus* egg extracts that cosediments with microtubules in an ATP-sensitive manner. In a *Xenopus* cell line, XKCM1 localizes to interphase nuclei and cytoplasm. During mitosis, a portion of XKCM1 relocates to centromeres and to spindle poles. To probe the function of XKCM1, we have used an *in vitro* assay for spindle assembly in *Xenopus* egg extracts. Immunodepletion of XKCM1 from extracts causes gross defects in spindle morphology and assembly. Microtubules quickly grow and encircle the sperm nuclei added to the extract to initiate the reaction. This central ring of microtubules then serves as an organizer for the formation of abnormally large microtubule asters which often contain microtubules four times longer than those in control extracts. With time, these asters aggregate together in massive structures containing multiple sperm nuclei. If XKCM1 antibodies are added to preformed spindles, there is rapid perturbation (< 2 min.) of spindle structure beginning with the assembly of long microtubules from the edges of the original spindle, and leading to the formation of massive aggregates similar to those seen in immunodepletion of XKCM1 prior to spindle assembly. These results suggest that XKCM1 is required for both establishment and maintenance of spindle structure. An intriguing possibility is that XKCM1 may control some global aspect of microtubule function such as regulating microtubule turnover. This unique role for a motor protein is consistent with the abundance of XKCM1, its localization to structures which interact with microtubule ends, and the rapidity of the effects of antibody inhibition experiments.

MOLECULAR MECHANISM OF CALMODULIN ACTION IMPLICATED IN YEAST ACTIN ORGANIZATION.

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Calmodulin is indispensable for cell proliferation. We isolated 14 temperature-sensitive mutants of yeast calmodulin that were recovered by systematically altering phenylalanine residues implicated in the target interaction¹. Based on the phenotypic analysis of the complementing calmodulin mutants, we proposed that calmodulin performs at least four essential functions including actin organization, nuclear division, bud emergence and calmodulin localization². The present study was undertaken to identify the protein ligand that participates in actin organization. The calmodulin mutant (*cmd1-226* : F92A) which impaired actin organization showed allele-specific synthetic lethal interaction with *myo2-66*, a mutation of a class V unconventional myosin. Myo2p is known as one of the essential calmodulin binding proteins in yeast³. A strain harboring *cmd1-226 myo2-66* double mutations did not grow at the permissive temperature for each single mutation. In contrast, all calmodulin mutations resulting in other functional defects showed no synthetic lethal interaction with *myo2-66*. Furthermore, a gel overlay assay indicated that a mutant calmodulin with the F92A alteration dramatically reduced binding activity to a GST-Myo2p fusion protein.

In order to obtain detailed information about the amino acid sequence requirements for the region of calmodulin important for actin organization, we used random replacement mutagenesis⁴. The region around the residue 92 was replaced with randomized sequences. Among over 50 replacement mutants, Phe, Val, Leu and Ile could be replaced at the position 92 and retained calmodulin activity. Ala, Trp were allowed, but resulted in a temperature-sensitive growth. Other amino acid residues were not recovered at this position in the none-lethal mutants. The pattern of the sequences that result in a functional protein indicated that hydrophobic and aromatic amino acid residues are allowed at this position. Taken together, hydrophobic interaction between calmodulin and Myo2p is important for regulation of actin network by calmodulin.

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THE MECHANICS OF MYOSIN SUBFRAGMENT 1

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Myosins are force generating enzymes that are thought to play many diverse roles in every eukaryotic cell. In the cell, conventional myosin exists as a two headed species consisting of two heavy chains, each of which binds two light chains. Furthermore, this two headed species forms multimeric filaments that are thought to be important for its function. We have been interested in whether single headed myosin species can accomplish the same mechanical feats that the two headed species can.

Hence, we have been studying the single headed subfragment 1 of myosin from *Dictyostelium discoideum*. This S1 is expressed in a strain that lacks an intact gene for the two headed species, which allows us to purify a single headed species unambiguously. In order to optimize the attachment of the protein to various surfaces we have added a *myc*-epitope tag to the C terminus, and attach the protein to surfaces using an anti-*myc* antibody.

Initial studies of the mechanics of single S1 molecules revealed that the step size and force generated are similar to full length myosin. Furthermore, an S1 without a regulatory light chain binding site produced smaller step sizes and larger forces than the full length S1. We discuss these results, and their implication for myosin function.

SITE DIRECTED MUTATION OF THE DICTYOSTELIUM MYOSIN ESSENTIAL LIGHT CHAIN SUGGESTS IT PROVIDES MORE THAN STRUCTURAL SUPPORT TO THE NECK REGION OF MYOSIN Guyu Ho, Tung-Ling L. Chen and Rex L. Chisholm. Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL.

In non-muscle cells, myosin II plays an important role in a variety of motility processes including cytokinesis, cell locomotion, chemotaxis and receptor capping. *Dictyostelium discoideum* has been an important experimental system for the investigation of cell motility, particularly the contributions of the acto-myosin system to non-muscle cell motility. *Dictyostelium* myosin II is a hexameric molecule consisting of two copies each of a 240 kd heavy chain (MHC), an 18 kd phosphorylatable or regulatory light chain (RLC) and a 16 kd alkali or essential light chain (ELC). We have used gene targeting to create null mutants which fail to express either light chain. These mutants exhibit defects in cytokinesis, development, cell locomotion and receptor capping. Re-expression of the missing light chain in the null mutants corrects these defects. It has been proposed that light chains contribute to myosin function by providing structural support for the helical portion of the MHC, thereby effectively creating a lever arm that extends the power stroke of the molecule. We have been using in vitro mutagenesis of the light chains followed by expression of the mutant light chains in the appropriate MLC null mutants to explore this hypothesis.

The essential light chain contains four helix-loop-helix domains. We have used an alanine scanning mutagenesis approach to convert charged residues in each of the loops into uncharged alanines. Mutations in L1, L2 and L3 bound with normal stoichiometry to the MHC when expressed in the ELC null background. When expressed in wildtype cells these mutants efficiently competed with the endogenous ELC for binding to the MHC suggesting that the affinity of their interaction with the heavy chain is comparable to wildtype. However, despite stoichiometric binding of ELC the cells still exhibited the defects in cytokinesis. Myosin purified from these cells exhibited decreased actin-activated ATPase and defects in motor function as assayed by an in vitro actin filament motility assay. These results suggest that the ELC contributes to myosin function in some way in addition to providing structural support for the alpha helical neck region of the MHC. Supported by NIH grant GM39264 to RLC.

CHARACTERIZATION OF CYTOPLASMIC DYNEIN IN DIFFERENTIATING CHICKEN ERYTHROBLASTS.

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Cytoplasmic dynein is a minus-end orientated motor protein with multiple cellular functions. It has been localized to membranous organelles and to the mitotic apparatus in a number of cell types. To get a better insight into the physiological role of dynein in specific intracellular transport processes, we used a chicken erythroblast cell line (HD3-E22) which can be induced to terminally differentiate in culture by turning off the function of the transforming oncogene (v-erbB). After induction of differentiation, the cells drastically upregulate expression of transferrin receptors (TfR). The abundance of endocytic structures is also greatly increased and the endocytic TfR cycle is altered in that a certain percentage of the Tf/TfR complex moves to more acidic endocytic compartments (endosomal carrier vesicle (ECV), late endosome (LE)). It has been demonstrated that ECV-LE interactions depend on cytoplasmic dynein. In order to examine the role of dynein in retrograde transport, we performed localization studies at the light and electron microscopic level using dynein subunit specific antibodies. We employed novel, monoclonal antibodies, the specificity of which has been characterized biochemically. These antibodies labelled vesicular structures in the juxtanuclear region of interphase cells, as well as spindle and kinetochores of mitotic cells. Immuno-EM localization studies in undifferentiated erythroblasts revealed a cytoplasmic labelling as well as staining of late endocytic structures. During differentiation, the localization of dynein changed drastically. One day after induction of differentiation the bulk of the protein was found in the ECV's, whereas the mature cells obtained after 5 days (which have stopped accumulating hemoglobin) exhibited a diffuse, cytoplasmic distribution of dynein. The presence of dynein in late endosomal compartments was confirmed with double labelling studies with an antibody specific for TfR as well as mannose-6-phosphate receptor (B. Hoflack, EMBL, HD). These results suggest that the cellular organization of dynein changes during erythroid differentiation, rendering the chicken erythroblast system a powerful model for studying the biological function of dynein in differentiating cells.

REGULATION OF ENDOPLASMIC RETICULUM MOVEMENT ALONG MICROTUBULES IN VITRO

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Many organelles within the cell can extend membrane structures using microtubule-based motors. Such membrane tubules can fuse to form extensive networks, as is seen for the endoplasmic reticulum. The formation of tubular membrane networks can be followed in vitro using extracts from *Xenopus* eggs, and this process has been shown to be under cell cycle control¹. The networks which form in interphase extracts are identified as endoplasmic reticulum by the presence of ER resident proteins, as shown by immunofluorescence, and by the presence of single ribosomes and polysomes, as shown by electron microscopy². ER movement in these extracts occurs towards the minus ends of microtubules, and this motility is abolished by treatment with vanadate or by UV-vanadate cleavage, suggesting that cytoplasmic dynein drives this ER motility. Phosphorylation of unknown components during metaphase results in the inhibition of ER movement and network formation¹. Increasing phosphorylation in interphase extracts using the phosphatase inhibitor okadaic acid, however, has a dramatically different effect on ER movement². Okadaic acid treatment results in an increase of up to 27-fold in the number of ER tubules moving and in the extent of ER networks formed. This activation is blocked by the broad-specificity kinase inhibitor 6-dimethylaminopurine. Okadaic acid has no effect, however, on the direction of ER tubule movement, or on the speed or duration of ER tubule extensions. In addition, soluble cytoplasmic dynein activity is not affected. The sensitivity of ER movement to okadaic acid closely matches that of protein phosphatase 1. Although the amount of ER motility is greatly increased by inhibiting PP1 in interphase extracts, the amount of cytoplasmic dynein associated with the membrane does not change². The data support a model in which phosphorylation regulates ER movement by controlling the activity of cytoplasmic dynein bound to the ER membrane.

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CHARACTERIZATION OF THE DYNEIN GENE FAMILY IN
CHLAMYDOMONAS REINHARDTII.

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A better understanding of dynein structure and function would be aided by establishing the relationships between the dynein genes and flagellar mutations. Using a PCR-based strategy along with DNA sequencing, we have identified eleven members of the dynein heavy chain (DHC) gene family in *Chlamydomonas*. Genomic Southern blot analysis indicates that each gene is single copy, and that there are a total of at least 12-13 genes in this family. Two products correspond to the alpha & beta DHC genes of the outer arm (Mitchell & Brown, 1994, J. Cell Sci. 107:635). Comparisons to cytoplasmic DHC sequences in the databases suggest that the remaining nine genes probably encode inner arm DHCs. Alignment of the predicted amino acid sequences spanning the nucleotide binding site indicates that these genes can be further subdivided into two or three groups. Reverse transcription of RNA followed by PCR with specific primers confirms that all of these genes are expressed. To correlate the DHC sequences with potential DHC mutations, we have placed each gene on the genetic map of *Chlamydomonas* using RFLP mapping procedures. Surprisingly, only one DHC clone is tightly linked to a known inner arm locus, although at least four other clones show linkage to other flagellar loci. As an additional strategy to identify DHC mutations, we are using our clones to analyze genomic DNA from new motility mutants generated by plasmid insertion (Tam & Lefebvre, 1993, Genetics 135:375). One mutant strain is the result of the insertion of the *ARG7* gene into the DHC1 locus. Approximately 12.5 kb of DNA spanning a region predicted to encode the four P-loops have been deleted from the genome of this mutant. Biochemical and structural studies reveal that isolated axonemes lack the I1 inner arm dynein. These results indicate that the DHC1 gene encodes one of the two heavy chains of the I1 inner arm complex. This is the first inner arm DHC locus to be identified in any organism. This approach should be a useful strategy for the identification of additional DHC loci and will aid in our further understanding of dynein isoform structure and function. (supported by grants from the NSF & March of Dimes to M. Porter).

LOCALIZATION OF THE *CHLAMYDOMONAS* KINESIN-LIKE
PROTEIN FLA10 AND ITS INVOLVEMENT IN MOTILITY
ASSOCIATED WITH THE FLAGELLAR MEMBRANE.

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The *Chlamydomonas FLA10* gene was shown to encode a flagellar kinesin-like protein (Walther *et al.*, *JCB* 126:175-188). By using a temperature-sensitive allele of *FLA10*, we have determined that Fla10 is necessary for both the bidirectional movement of polystyrene beads on the flagellar membrane and intraflagellar transport (IFT), the bidirectional movement of granule-like particles beneath the flagellar membrane (Kozminski *et al.*, *PNAS* 90:5519-23). 60-90 minutes after *fla10* cells were shifted to the restrictive temperature (32°C), we observed not only flagellar resorption, which is characteristic of *fla* mutants at 32°C, but a complete cessation of bead movement and IFT. At 30 minutes post-temperature shift, the amount but not the rates of each motility was already visibly reduced. Loss of IFT and bead movement was not observed in *fla10* cells induced to resorb their flagella by other treatments (e.g. IBMX), suggesting that flagellar resorption does not cause the cessation of either motility. Concomitant with the loss of IFT and bead movement, there was a 60-65% reduction in the number of electron dense complexes between the flagellar membrane and the axoneme, as assayed by EM thin-section. This correlation supports our hypothesis that the granule-like IFT particles observed by video-enhanced DIC microscopy are the electron dense complexes observed beneath the flagellar membrane by EM. No loss of electron dense complexes was observed in cells that maintained a normal level of IFT during flagellar resorption (e.g. IBMX treatment), suggesting that the presence of the electron dense complexes is independent of flagellar resorption. Additionally, by using affinity-purified antibodies raised against either a conserved kinesin motor domain peptide (LAGSE) or the non-conserved tail region of Fla10, we localized Fla10, in thin-sections of wild-type flagella, between the flagellar membrane and axoneme, the site where one would expect to find a motor involved with submembranous or flagellar surface motility. Supported by NIH grant GM 14642 and NSF grant 45147 to JLR.

MOLECULAR ULTRASTRUCTURE OF KRP₁₃₀, A BIPOLAR KINESIN RELATED PROTEIN FROM DROSOPHILA MELANOGASTER EMBRYOS. A.Kashina, R. Baskin, D. Cole, W. Saxton and J. Scholey. Section of Molecular and Cellular Biology, University of California at Davis, Davis, CA 95616.

We have used electron microscopy to analyze the structure of single molecules of the kinesin-related protein, KRP₁₃₀, purified from *Drosophila melanogaster* embryonic cytosol (Cole et al., J. Biol. Chem. 269:22913-22916, 1994). KRP₁₃₀ is thought to be a member of the bimC subfamily of kinesins which functions in spindle pole separation (reviewed by Saunders, Trends Cell Biol., 3:432-437), based on its immunoreactivity with an antibody specific for Eg5, another putative bimC family member, and on its slow, plus-end directed microtubule-based motility. Investigation of KRP₁₃₀ pelleted with microtubules by electron microscopy of both negatively stained and rotary shadowed specimens reveals bipolar molecules with a rod-shaped 'stalk' flanked by two globular domains on the ends. Some of the molecules appear bent in the middle indicating a 'hinge' region similar to that observed for conventional kinesin (Hirokawa et al. Cell 56:867-878, 1989). Investigation of both free molecules and molecules bound to microtubules showed that the globular domains are very similar to each other in size and shape; the globular domains associate with microtubules to form either linear protrusions from the microtubule wall, or crossbridges between two neighboring microtubules. The dimensions of the KRP₁₃₀ molecules are: mean length = 95.6 \pm 9.8 nm (n=131); mean diameter of globular domains = 21.7 \pm 3.7 nm (n=154); mean 'stalk' diameter = 9.6 \pm 1.2 nm (n=127); mean 'stalk' length = 61.3 \pm 8.3 nm (n=130). These results are consistent with our hypothesis that native KRP₁₃₀ is a bipolar tetramer composed of four identical 130 kDa subunits arranged as two antiparallel dimers with active 'motor' domains on opposite ends (Cole et al., 1994). Such bipolar tetramers could crossbridge antiparallel spindle microtubules emanating from two opposite spindle poles and cause them to slide apart, thereby driving spindle pole separation and participating in the formation of the mitotic spindle.

GENETIC INTERACTIONS BETWEEN KINESIN AND VOLTAGE-GATED ION CHANNELS. D. D. Hurd¹, M. Stern², and W. M. Saxton¹. 1) Department of Biology, Indiana University, Bloomington, Indiana, and 2) Department of Biochemistry and Cell Biology, Rice University, Houston, Texas.

To gain insight into cytoplasmic transport in general and into kinesin function specifically, we have been studying the effects of kinesin heavy chain mutations on *Drosophila*. *Khc* mutations cause two independent defects in neuronal physiology: reduced neurotransmitter secretion by nerve terminals and impaired compound action potential propagation by segmental nerves (Gho *et al.*, Science 258: 313). Selective staining of motor neuron terminals shows that *Khc* mutations cause marked reductions in terminal size. The short 6/7 neuron of larval segment A2 showed a 2.5-fold reduction in synaptic bouton numbers while the long 6/7 neuron of segment A6 showed a 4.4-fold reduction. This correlates well with the reductions in neurotransmitter secretion seen by electrophysiology for the same neurons: 2.7-fold for A2 and 4.7-fold for A6. Since synaptic boutons are the primary sites of neurotransmitter secretion, the reduced transmitter release seen in *Khc* mutants is probably due to the relatively small number of boutons. The simplest hypothesis for the dependence of terminal growth on kinesin function is that materials critical for the growth are delivered to the terminal by kinesin driven anterograde axonal transport. Other factors that could affect terminal growth include trophic interactions with the muscle and use-dependent plasticity.

The cause of the second physiological defect, impaired compound action potentials, is being studied using behavioral assays. We have found that *Khc* mutations cause temperature-induced paralysis, a defect that can be caused by mutations in *nap* and *para* that reduce the dosage of voltage-gated sodium channels. Tests of *Khc*-sodium channel double mutants show partial synthetic lethality and enhanced temperature induced paralysis. Mutations in the potassium channel subunits *Shaker* and *ether-a-go-go* cause spasmodic leg shaking in adult flies. The leg shaking is completely suppressed by *nap* or *para* mutations. We have found that *Khc* mutations also suppress the leg shaking of *Shaker* and *ether-a-go-go* mutants. All of these behavioral tests suggest that impaired kinesin function results in reduced inward sodium currents across axonal plasma membranes during action potential propagation. This supports the hypothesis that *Khc* mutations inhibit the delivery of voltage-gated sodium channels and/or other electrogenic proteins to the axon.

MICROTUBULE BEHAVIORS IN LIVING FIBROBLASTS DURING CHEMOTAXIS

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Microtubules are important to directed migration and cell polarization in many cell types, including fibroblasts. However, what role microtubules play and how they contribute to these morphogenic events is yet not clear. Here we attempt to investigate microtubule behaviors in living fibroblasts when they are subject to a concentration gradient of platelet-derived growth factor (PDGF) which is a chemotactic factor for many fibroblasts. We have designed a chemotaxis chamber by which we can grow cells on an inverted microscope stage and inject cells with rhodamine labeled tubulin before applying the gradient. The gradient formed in the chamber is stable for about a few hours as tested with fluorescein as a marker. With this chamber, we are able to make both phase and fluorescent time-lapsed movies using a cooled-CCD camera so the behavior of cells and microtubules can be visualized and correlated.

Preliminary studies have shown that many Swiss 3T3 fibroblasts observed have repolarized by sending out new lamellae toward high PDGF concentration and retracting old lamellae from the opposite side. The event of new lamella formation is accompanied by extensive membrane ruffling. Studies on microtubule dynamics are currently under way. Meanwhile, centrosome behaviors will also be monitored using rhodamine labeled tubulin. We hope these studies would provide us some new insights about how microtubules respond to an outside signal and how their behaviors correlate with cell motility.

DISTRIBUTION OF CALDESMON IN CHICKEN GIZZARD SMOOTH MUSCLES

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The distribution of caldesmon (CaD) was investigated at the cellular level as well as on isolated native thin filaments (NTF). Immunocytochemical examination indicated that both anti-myosin and anti-CaD show rather uniform distribution in relaxed muscle but exhibit banding patterns in slightly shortened muscle. In the latter, almost all myosin filaments were decorated with patchy clusters of anti-CaD, indicating close association of these two proteins. In contrast to those antibodies, the distribution of anti-tropomyosin (Tm) was uniform in both relaxed and shortened muscles. These results indicate that not all the actin filaments have CaD, suggesting two possibilities: 1) the presence of two types of filaments, either decorated with CaD or not; 2) CaD molecules clustered only at certain areas but not other areas on the same filaments. In order to improve the accuracy of identifying CaD in electron micrographs, actin and CaD in NTF were first crosslinked with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Graceffa et al., Biochem. J. 294:63, 1993), then Tm and uncrosslinked CaD molecules were washed away in 0.5 M NaCl and finally DTNB-treated NTF were examined with electron microscopy. Visualization of CaD molecules was aided with monoclonal anti-CaD for rotary shadowing and an additional gold-conjugated anti-antibody for negative staining. Both techniques indicated that the density of CaD molecules varies not only between different filaments but also along the same filament (Supported by grants from NIH).

CALPONIN, ITS ASSOCIATION WITH THE CYTOSKELETON AND POSSIBLE ROLES.

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With a method devised to preserve a high degree of antigenicity for post-embedding immunogold electron microscopy, the distribution of two smooth muscle proteins, caldesmon (CaD) and calponin (CaP), was examined. Although both of these two proteins are known to bind actin filaments and inhibit the in vitro actomyosin ATPase activity, their in vivo distributions were quite different. The distribution of anti-CaD appeared to be narrower than that of anti-tropomyosin, and overlapped extensively with myosin filaments in shortened muscles, indicating that CaD recognizes a subset of thin filaments that are near myosin filaments; this is consistent with the hypothesis that CaD plays a regulatory role in smooth muscle contraction by modulating the interaction between actin and myosin. The distribution of anti-CaP, on the other hand, was quite different from that of anti-CaD or anti-tropomyosin. Most of CaP was found, not in the vicinity of myosin filaments, but to be associated with the cytoskeleton. Co-localization studies of CaP with other cytoskeletal proteins, such as desmin and β -actin, indicate that while CaP exhibits partial overlapping with all these proteins, its pattern is more similar to the distribution of desmin than that of β -actin. Thus our observations argue against CaP being directly involved in the regulation of actomyosin interaction during contraction, and raise the possibility that CaP may bridge various cytoskeletal proteins and/or interface the cytoskeleton and contractile elements, and thereby play a role in the regulation of cytoskeleton disassembly and re-assembly in smooth muscle cells. Supported by a grant from NIH (AR-41637).

SORTING AND TARGETING OF LAMIN B-CONTAINING VESICLES DURING MITOSIS

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In all higher eukaryotic cells the nuclear envelope is reversibly disassembled at the onset of mitosis. In somatic cells, it is generally thought that A-type lamins disperse as soluble oligomers in the mitotic cytoplasm, whereas B-type lamins remain attached to membrane vesicles. Whether lamin-carrying vesicles or soluble lamins are involved in post-mitotic nuclear reassembly is currently debated. To address key aspects of this problem, we have used morphological and biochemical approaches in combination with *in vitro* reconstitution assays. Employing Streptolysin O-permeabilized cells, we have shown that the bulk of B-type lamins occur in a non-diffusible state and are associated with vesicular structures throughout mitosis. In contrast, A-type lamins appear to be partly soluble and partly associated with the surfaces of chromosomes, forming a "minimal lamina". Extending previous observations, we now report that lamin B-containing vesicles co-localize with vimentin filament bundles during prometaphase, but detach from the filaments in late anaphase and assemble around chromosomes at telophase. Lamin B-carrying vesicles, immunoisolated from prometaphase cells *en bloc* with vimentin filaments, can specifically capture chromosomes. Efficient chromosome capturing requires the presence of cytosol, is dependent on dephosphorylation and can be inhibited by microcystin and okadaic acid. Stripping of peripheral membrane proteins by urea extraction abolishes binding of the vesicles to chromosomes. However, reconstitution of urea-stripped membranes with purified B-type lamins restores their ability to bind to chromosomes in a cytosol and phosphorylation-dependent fashion. Lamin B-reconstituted vesicles can fuse into large cisternae on the surfaces of chromosomes, but do not form a continuous membrane "shell" around chromatin. These observations suggest that the vimentin-associated vesicles represent precursors of the nuclear envelope. Apparently, targeting of mitotic vesicles to chromosomes requires the presence of B-type lamins and is regulated by protein phosphatases and cytosolic factors.

APPLICATION OF 400 kV CRYO-MICROSCOPY AND SEMI-AUTOMATED COMPUTER PROCESSING TO THE ANALYSIS OF ACTIN FILAMENT STRUCTURES

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Most three-dimensional reconstructions of filamentous actin have been limited to 20 to 30 Å resolution despite the fact that resolving power of modern transmission electron microscopes exceeds 2 Å. There are several factors which contribute to this limitation including the inherent disorder in actin filaments and the relatively weak diffracting power of helical structures.

In some cases we and others have been able to extend the "effective resolution" of actin structures by incorporating atomic models into the electron density maps obtained by electron microscopy. Nonetheless, in most instances atomic structures are not available or, even if they are available, the resolution is not sufficient to permit unambiguous fitting of the atomic structures into the EM map. There is a clear need, therefore, to modify both the data collection and analysis of filamentous actin structures.

We have begun a two-pronged approach to improve the resolution of our reconstruction of actin filaments decorated with α A1-2, the actin-binding domain of alpha-actinin (McGough *et al.*, 1994, JCB 126:433). The first step is to use cryoelectron microscopy at intermediate voltage (400 kV) thereby improving on the quality of our electron micrographs. The second step involves the application of semi-automated procedures to the analysis of the EM data. The initial goal is to reconstruct the filaments at sufficient resolution to resolve the two subdomains comprising α A1-2.

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γ-TUBULIN IS AT THE MINUS ENDS OF SPINDLE MICROTUBULES
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γ-tubulin appears to play an essential role in the cellular initiation of microtubules (Mts). Initial studies showed γ-tubulin associated with the centrosomal material of fungi and vertebrates. More recent work has, however, suggested that γ-tubulin may become associated with the walls of spindle microtubules (Mts): immuno-localization with antibodies to γ-tubulin has shown spindle fiber staining (Liu et al., J.Cell Sci. 104: 1217['93]; Lajoie-Mazenc et al., *ibid* 107:2825['94]). Anti-γ-tubulin also stains the pole-proximal ends of midbody MTs (Julian et al., *ibid* 104: 145['93]), suggesting that non-centrosomal sites may function for MT initiation during telophase in vertebrates as in *S. pombe* (Horio et al., *ibid* 99:693['91]).

3-D reconstructions of mitotic spindles from PtK cells in metaphase and anaphase have shown that the interpolar MTs (IPMTs) are positioned with their minus ends farther from the pericentriolar material than expected (Mastronarde et al., J.Cell Biol. 123:1475['93]). We surmised that these minus ends might be associated with γ-tubulin and thereby account for the unexpected mitotic distributions of γ-tubulin cited above. EM immunocytochemistry with several affinity purified antibodies has revealed a distribution of γ-tubulin stain relative to the spindle poles that is quantitatively like that of the IPMT minus ends. Preliminary work on the 3-D localization of spindle γ-tubulin by EM suggests that it is situated near the minus ends of MTs, including the IPMTs that lose their association with the pericentriolar material and move out into the kinetochore MT bundle. By telophase, all IPMTs in PtK cells have dissociated from the centrosome, so their minus ends are free, accounting for the γ-tubulin localized at the pole-proximal ends of the midbody. Kinetochore MTs, on the other hand, do not detach from the spindle poles, suggesting that the pericentriolar material distinguishes these polymers and makes different linkages with them from metaphase through anaphase. The implications of this suggestion for mitotic mechanism will be discussed.

DEVELOPMENTAL EXPRESSION OF A KINASE ANCHOR PROTEINS (AKAPs) IN RAT TESTIS

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cAMP is important for the initiation of flagellar motion. We have shown that a type II cAMP-dependent protein kinase (PKA) is tightly associated with rat sperm flagella via its regulatory (RII) subunits. It has been proposed that subcellular distribution of PKA may be dictated, in part, through association with A Kinase Anchor Proteins (AKAPs). AKAPs are multi-functional proteins which tether the kinase to cytoskeletal structures or specific organelles. Using an RII-overlay procedure, we have shown that RII α can associate specifically with rat sperm flagellar polypeptides of 57, 80 and 120 kDa. These polypeptides were present on the fibrous sheath. The 57 kDa polypeptide was identified as RII. RII-binding proteins of 80 and 120 kDa were also present in particulate extracts of adult rat testis. Developmental studies using the RII-overlay indicated that both testicular AKAPs are only expressed at late stages of spermatogenesis, i.e. during spermiogenesis. cDNAs encoding AKAPs were cloned from a rat testis λ gt11 expression library using an RII-overlay procedure. Clones encoding three distinct AKAPs (TAKAP 0.8, TAKAP 1.2 and TAKAP 2.2) were isolated and shown by sequence analysis to be unrelated. Northern blot analysis revealed that TAKAP 0.8 and TAKAP 1.2 hybridized with testis-specific transcripts expressed at late stages of spermatogenesis. The mRNAs detected were 5.5 kb and 1.8 kb for TAKAP 0.8 and TAKAP 1.2, respectively. TAKAP 2.2 hybridized with a 9.5 kb mRNA which was expressed at all stages of development. TAKAP 0.8 and TAKAP 1.2 cDNAs were inserted into the pRSET vector and expressed in *E. coli*. Lysates from the bacteria and recombinant proteins purified on nickel columns and reverse phase HPLC were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters and probed with RII α . The expressed proteins bound RII indicating that the cDNAs encoded the RII-binding domains of these AKAPs. Antiserum was prepared against the purified TAKAP 1.2 polypeptide. Western blot analysis showed that the antiserum recognized an 80 kDa polypeptide present on isolated rat sperm fibrous sheath. Additional studies to determine the entire structure of these AKAPs and how they are assembled into the fibrous sheath are in progress.

GLUTAMATE-INDUCED REGULATION OF TAU EXPRESSION IN PRIMARY CULTURE OF CEREBELLAR NEURONS.

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We found that primary cultures of cerebellar granule cells from 8 day neonatal rats express at least two isoforms of tau protein mRNA that are distinguishable for the presence of three or four internal repeats. On the basis of their differential pattern of expression during development, the two different mRNA isoforms have been named as foetal (three repeats) and mature (four repeats) tau mRNA. Synthesis of tau proteins was found to be a developmentally regulated process affecting different levels of tau expression including changes of tau RNA splicing and translation. Indeed, our data suggest that the differential expression of various tau proteins parallels the degree of cell maturation *in vitro* and is apparently regulated by neuron-to-neuron contacts.

A brief pulse of either toxic or subtoxic concentrations of glutamate to primary culture of rat cerebellar granule cells induced a short lasting accumulation of both isoforms of the mRNA for tau proteins. Time course experiments revealed a bell-shape curve with the maximal increase of tau mRNA levels 2 hr after the glutamate pulse. Tau mRNA is further processed to protein as suggested by the significant increase of tau immunoreactivity detectable 2 and 4 hr after the pulse.

Pretreatment of granule cells with an antisense oligonucleotide complementary to 26 nucleotide sequence comprising the ATG translation initiation codon of tau gene completely prevented the increase of tau immunoreactivity induced by glutamate. A significant amount of the tau antisense oligonucleotide (about 1 to 2 % over total) was taken up by the cells and remained stable into the cells for at least 60 min. A dose response study revealed that 25 μ M tau antisense oligonucleotide was the most efficacious concentration in terms of prevention of glutamate-induced tau immunoreactivity increases, without affecting basal tau expression. Higher concentrations of tau oligonucleotide antisense reduced tau immunoreactivity in control cells. Significantly, the concentration response curve of glutamate for inducing neuronal death in cells pretreated with tau antisense oligonucleotide showed a shift to the right when compared to those obtained in untreated or tau sense oligonucleotide-treated cells.

EXPRESSING FUNCTIONAL DOMAINS OF MOUSE CALPONIN

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Smooth muscle calponin (CaP) is a 34 kDa protein that binds to actin-, tropomyosin and Ca^{2+} -binding proteins and inhibits the actomyosin ATPase activity as well as the movement of actin filaments over coated myosin in *in vitro* motility assays. Previous work (Mezgueldi et al., 1992, J. Biol. Chem., 267, 15943-15951) reported indirect evidence for the binding of F-actin to the 38-residue stretch of gizzard calponin encompassing the sequence A145-Y182 and postulated the hexapeptide motif VKYAEK, representing residues 142-147, as the putative actin-binding site. We investigated the direct influence of this domain on actin binding as well as on actomyosin ATPase inhibition by expressing in *E. coli* various fragments of mouse calponin embracing amino acids 1-228 (CaP1-228), 45-228 (CaP45-228), 131-228 (CaP131-228) and CaP1-228 with substitution of Ala145 with Ser (CaP1-228/A \rightarrow 145S) or deletion of Val142-Lys147 (CaP1-228/Del142-147). The smallest recombinant calponin fragment, corresponding to the sequence Ala131-Ile228 and comprising the hexapeptide motif, was found to retain actin-, calmodulin- and tropomyosin-binding activity. Most importantly, its interaction with actin occurred competitively with the binding of native calponin and inhibited the actomyosin ATPase activity in a Ca^{2+} -calmodulin-regulated fashion. In addition, insertion of the hexapeptide motif into the closely sequence-related protein SM22 failed to confer any noticeable actin binding abilities to this protein.

Thus, the overall sequence 131-228 contains the major structural determinants for the regulatory actin-binding and inhibitory activity of calponin. Modulation of the hexapeptide motif either by replacement of Ala145 with Ser or by deletion of the entire motif had no effect on calmodulin- or tropomyosin-binding but reduced in both cases the actin affinity. However, while the Ala145 \rightarrow Ser mutant inhibited less efficiently the actin activation of myosin Mg-ATPase, the mutant CaP1-228/Del142-147, missing the VKYAEK sequence had no inhibitory activity. These data indicate that the hexapeptide VKYAEK is crucial for the inhibition of the actin activated myosin ATPase activity by calponin, but represents only a part of the actin-binding domain. Computer assisted structural prediction methods further demonstrate, that the regions carrying functional domains represent areas of high structural conservation in calponin.

MOLECULAR CLONING OF α -TUBULINS FROM *NEUROSPORA CRASSA*, TRANSCRIPTIONAL REGULATION, AND OVER EXPRESSION OF RECOMBINANT PROTEINS IN *E. COLI*.

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In most eukaryotes the tubulin genes comprise small multigene families with approximately equal numbers of genes for α and β -tubulin, the structural proteins of microtubules...We have isolated a full cDNA of α -Tub B and a partial cDNA of α -Tub A. Southern blot analysis at low stringency has shown that there are only two genes coding for α -tubulin in *N. crassa*, whereas three isoforms of α -tubulin have been detected. We have shown that the level of Tub A and Tub B transcripts increases during the first six hours of conidial germination, then decreases gradually for the next ten hours. Tub A is not transcribed in dormant macroconidia and in the first thirty minutes of germination, whereas Tub B transcript is present during all the developmental course of *N. crassa*. We have overexpressed the fusion proteins in *E. coli*. The recombinant proteins are recovered in inclusion bodies. We are currently trying to purify α -Tub B to attempt to refold it, and we plan to induce the polymerization of the heterodimer with β -tubulin in protofilaments.

IDENTIFICATION OF NOVEL FISSION YEAST
CYTOSKELETAL REGULATORY FACTORS INVOLVED IN
THE MITOSIS TO INTERPHASE TRANSITION.

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The evolutionarily conserved nuclear GTP-binding protein Ran has been implicated in several cellular processes, including cell-cycle progression, mRNA transport, DNA replication, and nuclear protein import. Ran is converted to its active GTP-bound state by the chromatin associated protein RCC1 and is inactivated by the GTPase activating protein RNA1. The downstream effectors of Ran that link the GTPase to its cellular functions have not yet been identified. The fission yeast homologs of Ran (named *spi1*) and RCC1 (named *pim1*) have been cloned and conditional mutants in *pim1* have been isolated. These mutants display a cell cycle arrest at the mitosis to interphase transition. The *pim1* mutant cells are binucleate, septated, and have fragmented nuclear envelopes.

We are analyzing additional mutants that show certain phenotypic similarities to the *pim1* mutant. These mutants can be rescued by expression of *spi1* but not *pim1*, suggesting they are either mutated in *spi1* or in factors downstream of the GTPase. One mutant of particular interest arrests as a four cell filament with four individual nuclei separated by three septa. Identification of the gene mutated in this strain is currently underway. Additionally a number of cDNA clones have been isolated from a library that rescue this mutant only when overexpressed. Two of the cDNAs show strong sequence similarity to factors required for proper formation of actin filaments and mitotic spindles in budding yeast. The genetic link between the *spi1* GTPase and these cytoskeletal factors will be addressed.

KINESINS WITH DIFFERENT MOTILITY REQUIREMENTS.

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Free-living amoebae exhibit abundant organelle movements, which depend both on microtubules (MTs), kinesin, and dynein. In flattened amoebae examined by video-DIC-microscopy, minute vesicles, mitochondria, and low-refractive-index vacuoles move bidirectionally along single MTs. The movements disappear when cells are treated with colchicine. Organelle translocating activity remains active for several hours after homogenization in 2-10 volumes of low salt motility buffer (15 mM imidazole, pH 7.5, containing 2 mM EGTA, 1 mM Mg-ATP and 0.6 M mannitol, an osmolyte for protecting organelles), and depletion or inactivation of one of the motors with antibodies leaves the activity of the remaining motor intact, allowing one to study the properties of just one motor in vitro. These movements are characterized by the following properties: (1) Organelles in vitro move at slower rates (0.4 $\mu\text{m/s}$ for plus-end kinesin movements, and 2 $\mu\text{m/s}$ minus-end dynein movements) than they do in vivo (2-5 $\mu\text{m/s}$). The in vitro rates are similar to the velocities measured for purified kinesin and dynein by video microscopy; (2) Although both mitochondria and small vesicles exhibit robust motility in vivo, mitochondria are only rarely observed to move in vitro, where MT-dependent motility is mostly exhibited by small microsomal vesicles. These differences appear to correlate with unique salt and ATP optima that are characteristic of amoeba kinesin.

Previously, we showed that bovine brain kinesin supports efficient movements of plastic beads and neurosecretory organelles in low ionic strength buffer. Analysis of the ATPase mechanism revealed that the K_m for ATP remained unchanged (~ 0.1 mM), while the K_{app} for MTs decreased from a high of 2 μM in low salt buffer to 0.75 μM in the presence of 50 mM NaCl. Thus, bovine brain kinesin responds to ionic strength much like skeletal muscle myosin, in that both motors increase their motility and ATPase activity affinity for polymer lattices in response to decreasing ionic strength. Thus, the motor-dependent movement of organelles can be studied to advantage by simply decreasing the ionic strength of the buffer. In contrast, amoeba kinesin ATPase and motility were maximal at 100-150 mM KCl. Furthermore, organelle velocity increased back up to the in vivo rate of 2-4 $\mu\text{m/s}$. These studies demonstrate the importance of optimizing the affinity of kinesin for MTs for reactivating organelle motility in vitro.

REGULATION OF THE ABILITY OF ELONGATION FACTOR 1 ALPHA TO INHIBIT ACTIN POLYMERIZATION J. W. Murray, B. T. Edmonds, G. Liu, and J. Condeelis. Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx NY, 10461

Measurements in rabbit reticulocytes indicate that Elongation Factor 1 alpha (EF1a), the essential component of the protein synthetic machinery, is in 14 fold molar excess to tRNA¹. This abundant protein also appears to interact with many factors other than the protein synthetic machinery including microtubules^{2,3}, calmodulin^{2,4}, and phosphatidylinositol 4-kinase⁵ and actin. EF1a binds and bundles F-actin and is colocalized with F-actin in vivo. We have previously demonstrated that EF1a prevents the fast growing ends of actin filaments from polymerizing or depolymerizing by a mechanism which resembles barbed end capping⁶. Recognizing that many factors can interact with EF1a, we are seeking to understand which factors can regulate this capping activity. Previous evidence from our lab has shown that GTP reduces the affinity of EF1a to actin crosslinked to sepharose beads⁷. While the addition of GTP-gamma-S to saponin permeabilized neutrophils has been shown to induce rapid actin polymerization indicative of an uncapping of filament ends⁸. The experiments presented in this poster examine the effect of various agents on the ability of EF1a to inhibit actin polymerization and, in some cases, bundle F-actin. Results indicate that EF1a capping activity is dependent on pH. Below pH 6.8 the capping activity may block up to 80% of the actin polymerization while above pH 7.2 the capping activity is near zero. In addition many factors including high salt, guanine nucleotides, and calmodulin can reduce the ability of EF1a to inhibit actin polymerization and can affect F-actin bundling. These data suggest signal transduction pathways by which EF1a could alter the rates of actin polymerization in vivo.

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CAP Z IN RESTING AND THROMBIN ACTIVATED PLATELETS. Vivianne T. Nachmias and Rajasree Golla, Department of Cell and Developmental Biology and Pennsylvania Muscle Institute, School of Medicine, University of Pennsylvania, Philadelphia, PA. 19104-6058

We recently reported (Mol. Biol. Cell **5**, 272a, 1994) the presence of cap Z at a minimum level of 0.2 micromolar as determined by immunoblotting SDS denatured resting human platelets (RHP) using chicken muscle cap Z as a standard.

Association of some platelet cap Z with actin filament ends is supported by the following results: ~25% is in the low speed cytoskeletons (CSK); 20 μ M phalloidin added at lysis brings down 30-60% more cap Z (but not myosin); and ~50% of the cap Z (but not radixin) is chased from RHP CSKS when shaken for 2 hrs. at 5°C with gelsolin-actin, a high affinity capper. Capping activity and cap Z were detected in the eluted, renatured 39-30 kDa section of an SDS gel and no activity in a control section.

We now report, from immunoblots of cytoskeletons and very high speed supernatants (VHSS) of platelets lysed 10 seconds after 2U/ml thrombin, a translocation of cap Z from CSK to the VHSS (90,000 rpm 1 hr, 320,000 g, $k = 6$) as compared to controls. As $t \text{ (hrs)} = k/s$ cap Z associated even with actin dimers should have sedimented; actin dimers have $s \sim 4.8-5$ (Bubb et al. JBC **269**, 25592, 1994) and the s value of cap Z is 4.8-4 (Cooper et al. JCB **99**, 217, 1984; Cassella et al., JBC, **261**, 10915, 1986). Also, the cap Z bands are not associated with the actin region on immunoblots of 2-D native-SDS gels of VHSS from 10 sec thrombin stimulated platelets. An attractive hypothesis is that part of the increased cap Z in the VHSS is due to uncapping. On conventional 2-d gels we find two major and two minor beta isoforms of cap Z and two alpha isoforms. We are testing whether a specific isoform is involved in the translocation of cap Z.

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INTERACTION OF THE INTEGRIN $\beta 4$ SUBUNIT AND THE HEMIDESMOSOMAL PLAQUE PROTEIN HD1

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Hemidesmosomes are adhesion structures by which epithelial cells adhere to the underlying basement membrane and which serve at their cytoplasmic face as anchorage sites for the keratin filaments. The integrin $\alpha 6 \beta 4$ is a major component of hemidesmosomes and plays an important role in the assembly of these structures, although the protein interactions necessary for assembly are far from clear. The $\beta 4$ subunit is distinguished from the other integrin β subunits by its large cytoplasmic domain of 1000 aa whereas the other β cytoplasmic domains contain around 50 aa. The only known protein motifs present in this cytoplasmic domain are four fibronectin (FN) type III repeats which reside in two pairs separated from each other by the intervening segment. The first pair of repeats and the intervening segment have been shown to be essential for incorporation of the $\alpha 6 \beta 4$ integrin into hemidesmosomes and it is thought that this REGION interacts with the keratin filaments, either directly or via linker proteins present in the hemidesmosomal plaque. To identify proteins that bind to the cytoplasmic domain of the $\beta 4$ subunit, a GST-fusion protein consisting of the two pairs of FN type III repeats and the intervening segment, was expressed in bacteria. A 500 kD protein was precipitated by the GST-cyto $\beta 4A$ fusion protein and identified as the HD1 protein. A GST-cyto $\beta 4B$ protein, which has 53 aa inserted in the intervening segment, also bound HD1. The $\beta 4B$ subunit is a minor variant on cells which form hemidesmosomes but is also found on other cell types that do not form hemidesmosomes but do express HD1. Indeed, we found that transfection of the $\beta 4B$ cDNA into rat 804G bladder carcinoma cells, resulted in association of $\beta 4B$ with endogenous $\alpha 6$ and was efficiently incorporated into hemidesmosomes, where it colocalized with HD1. We suggest that HD1 is involved in linking the $\alpha 6 \beta 4$ integrin to the keratin filaments.

LINKING MICROFILAMENTS TO INTRACELLULAR MEMBRANES: THE ACTIN BINDING AND GOLGI ASSOCIATED PROTEIN COMITIN EXHIBITS A MANNOSE SPECIFIC LECTIN ACTIVITY

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Comitin is a 24 kDa actin binding protein from *Dictyostelium discoideum* that is present on Golgi and vesicle membranes ¹⁻³. We have used a series of truncation mutants to investigate the molecular basis of these interactions. Comitin dimerizes in solution and its primary actin binding activity appears to be located between residues 89 and 135. Comitin and several plant lectins share a significant homology and, moreover, the F-actin crosslinking activity of comitin and its binding to vesicles can be inhibited by the addition of mannose. It appears that comitin's binding to membrane vesicles is mediated via an interaction of its NH₂-terminal "core" domain with mannose residues on surface glycoproteins or glycolipids. Brefeldin A which affects the integrity of the Golgi as well as cytochalasin A which disturbs the actin cytoskeleton both led to a significant change in the cellular distribution of comitin. These data indicate that comitin binds to both the Golgi and transport vesicles via mannose residues and, by way of its interaction with actin, links these membranes to the cytoskeleton.

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STRUCTURE OF THE TEK TIN-TUBULIN PROTOFILAMENT RIBBON. Dana Nojima, Richard W. Linck, and Edward H. Egelman
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Doublet microtubules contain an A- and B-tubule, composed of 13 and 10 protofilaments, respectively, all of which are thought to be made of tubulin. Tektins, a set of microtubule associated proteins found in the axoneme, are of structural interest because of their stability during the extraction of doublet microtubules and their localization near to where the B-tubule attaches to the A-tubule and near the binding sites of radial spokes, inner dynein arms and nexin links. We have fractionated doublet microtubules from sea urchin sperm flagella into ribbons of stable protofilaments, which can be shown to originate from the A-tubule. Using cryo-electron microscopy, conventional electron microscopy, scanning transmission electron microscopy, three-dimensional reconstructions and kinesin decoration, we have found that one protofilament of the tektin-tubulin ribbon is not composed of tubulin. Contrary to the widely assumed model, at least one protofilament in the wall of the A-tubule is not tubulin. Our findings suggest that this non-tubulin protofilament is primarily composed of tektins. A 480 Å axial periodicity within these ribbons can be related to the structure of tektin, and may determine the large-scale structure of the axoneme in terms of the binding of dynein, nexin and radial spokes to the doublet microtubule.

CYTOPLASMIC DYNEIN IS ATTACHED TO SORTING ENDOSOMES DURING RECEPTOR-MEDIATED ENDOCYTOSIS.

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In studies of receptor-mediated endocytosis (RME) of asialoorosomucoid (ASOR) by cultured rat hepatocytes, we previously demonstrated that receptor-containing endosomes bind to microtubules in an ATP-insensitive manner, while ligand-containing endosomes bind in an ATP-sensitive manner, presumably because they are attached to and move along the microtubules by cytoplasmic dynein (Goltz *et al.*, 1992). In an *in vitro* assay, when ligand-containing endosomes are released into the supernate by ATP, they can then be immunoprecipitated by antibodies to cytoplasmic dynein (Oda *et al.*, 1995). These results are consistent with two models of sorting: where (1) cytoplasmic dynein binds to the sorting endosome and could participate in sorting or (2) cytoplasmic dynein binds to the ligand-containing endosome only after sorting has occurred. To distinguish between these alternatives, we have treated the cultured cells with 50 μ M monensin for up to 60'. Although ASOR-binding to microtubules decreases dramatically from 15'-60' after initiation of single wave RME in control cells, because ligand is moved to lysosomes and degraded, the amount of ASOR binding in the presence of monensin remains constant, because sorting is inhibited. Sorting endosomes containing receptor and ASOR were removed from microtubules after the 60' monensin treatment. The vesicles were then immunoprecipitated by antibody to receptor or to cytoplasmic dynein. In each case, ¹²⁵I-ASOR was coprecipitated. Treatment with NP40 showed that ¹²⁵I-ASOR was in endosomes. Similarly, immunoprecipitation by anti-dynein coprecipitated receptor. In untreated controls at 60', cytoplasmic dynein and receptor do not coprecipitate.

We conclude that cytoplasmic dynein is bound to the sorting endosome after 15' of single wave RME and, therefore, is in a position to function as a motor in sorting.

SUCCESSIVE ASSEMBLY OF TWO PROTEINS ONTO THE CENTROSOME COORDINATED WITH SPECIFIC EVENTS DURING MITOSIS.

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CP190 (Centrosomal Protein of 190 kDa, originally called DMAP 190) was previously identified using a combination of microtubule affinity chromatography and immunocytology. Antibodies made to this protein stain the centrosome during part of the cell cycle in *Drosophila* embryos; nuclear staining during interphase is also apparent. Immunoaffinity chromatography experiments using affinity purified anti-CP190 antibodies identified a group of interacting proteins. One of these is CP60 (previously called DMAP 60). Antibodies to CP60 also stain the centrosome and nucleus. We have characterized the cell cycle dependent localizations of CP60 and 190 in greater detail. 3D microscopy was done in live embryos injected with fluorescently labeled 6-His fusion proteins derived from CP60 and CP190. In late interphase, both CP 190 and 60 are found in the nucleus. Labeled CP190 and 60 have each been coinjected with a fluorescently labeled 40,000 MW dextran, which is excluded from nuclei, to determine when their transition from the nucleus to the centrosome occurs relative to nuclear envelope breakdown. Recruitment of CP190 to centrosomes appears to occur immediately following nuclear envelope breakdown and it achieves its maximal intensity by metaphase; CP60 remains in the nucleus until it assembles onto centrosomes at the metaphase to anaphase transition. We have also stained fixed embryos from the syncytial nuclear cycles 10-14 for γ -tubulin, CP60, CP190 and DNA simultaneously. Combining the data from the fixed and live experiments, we have constructed a detailed picture of the localization of these proteins throughout the nuclear cycle during these divisions. We have also characterized the nuclear localizations of these two proteins in 3D at high resolution. CP60 and CP190 appear to form fibrous networks within the nucleus which do not colocalize with each other or with DNA; both CP60 and CP190 also biochemically fractionate with nuclear matrices. We have followed the transition of CP60 and CP190 from nuclei to centrosomes in the presence of colchicine- demonstrating that MTs are not necessary for CP190 or CP60 to attain or maintain their centrosomal localizations.

EXPRESSION OF MAMMALIAN MAP MICROTUBULE BINDING
DOMAINS RESULTS IN ABERRANT CELL DIVISION IN THE
FISSION YEAST, *S. POMBE*

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MAP 4 is a ubiquitous microtubule-associated protein which appears to play a role in mitosis. To investigate whether regulation of this protein might be accessible for study in a genetically tractable system, constructs of MAP 4 were transformed into the fission yeast, *Schizosaccharomyces pombe*. Full-length MAP 4, the microtubule binding domain (MTBD) and the MAP 4-specific portion of the binding domain (PSP; West et al. 1991. JBC 265:21886) were inserted in both orientations into pREP3X; this plasmid carries the leu-2 marker and a thiamine-repressible promoter (NMT). Growth and morphological phenotypes equivalent to wild type cells were seen for all transformants containing reverse orientation constructs, whereas a range of phenotypes was seen with the transformants bearing the sense constructs. Following growth in the absence of thiamine, transformants with full length MAP 4 had doubling times and morphologies that were normal. In contrast, those bearing the MTBD displayed multiple phenotypic abnormalities: cells were branched, abnormally long, or had multiple septa. In the majority of the cells, DNA was unevenly distributed or segregated, and a high percentage were aploid. Tubulin staining showed that spindles appeared normal, but were often asymmetrically placed. Cytoplasmic microtubules were variously arranged. In highly elongate cells, the interphase MTs ran throughout the length of the cells, whereas in short abnormally symmetric cells, microtubules were bundled and packed in the cytoplasm at high density. The MTBD transformants had slower growth rates than wild-type, but these phenotypes were not lethal over 50 generations in liquid culture, suggesting that some of the defects can be compensated. PSP transformants also showed abnormal morphologies, but these were less severe than those observed with the MTBD, and consisted largely of rounded cells with abnormally distributed DNA; cells that were multi-septate or branched were not seen. These data suggest *S. pombe* is similar to mammalian cells in showing a perturbation of MTs by MAP 4 binding domains but not by full-length protein. The basis for the regulation of these interactions will now be analyzed by genetics. (Supported by NIH GM22214 and NSF GER 9350145 to JBO and NIH GM 36663 to JRMcl.)

LOCALIZATION OF CALPONIN RELATIVE TO THE α AND β ISOFORMS OF ACTIN IN RESTING AND STIMULATED SMOOTH MUSCLE CELLS

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Calponin is a thin filament-associated protein that has been implicated to play an auxiliary role in regulation of smooth muscle contraction. In our previous work we have used immunofluorescence and digital imaging microscopy to determine the cellular distribution of calponin in single cells freshly isolated from ferret portal veins (Parker et al., *Am. J. Physiol.*, **267** (Cell Physiol. **36**): C1262-C1270, 1994). The results show that in resting cells calponin is distributed throughout the cytosol, associated with filamentous structures. The ratio, R , of surface cortex-associated calponin to cytosol-associated calponin was found to be 0.64 ± 0.02 . This distribution did not change upon depolarization of the cell with physiological saline containing 96 mM K^+ ($R=0.68$). Upon stimulation with an agonist (10 μ M phenylephrine) that is known to activate protein kinase C, the distribution changed from primarily cytosolic to primarily surface cortex-associated ($R=1.24$). In the present work we attempted to determine the structural basis for this redistribution by relating it to the distribution of actin isoforms. In resting cells α -actin immunofluorescence co-localized with filaments that run parallel to the long axis of the cell throughout the cell ($R=0.80$). In contrast β -actin immunofluorescence was discretely distributed near the surface membrane ($R=1.12$). Neither distribution changed significantly upon stimulation with phenylephrine. As found previously, R for calponin increased from 0.64 to 1.18 upon agonist stimulation. In cells co-labeled for both proteins the percent overlap of calponin and α -actin decreased significantly upon stimulation (59% to 35%), while that between calponin and β -actin increased significantly (42% to 63%). Thus the distribution of calponin appears to similar to that of α -actin in resting cells, and to that of β -actin in stimulated cells, suggesting that agonist stimulation causes a preferential redistribution of calponin from α -actin to β -actin. (Supported by NIH HL31704, HL42293, P01-AR41637)

ALTERNATIVELY SPLICED EXONS OF THE β -TROPOMYOSIN GENE EXHIBIT DIFFERENT AFFINITIES FOR F-ACTIN AND EFFECTS WITH NONMUSCLE CALDESMON

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The rat β -tropomyosin (TM) gene expresses two isoforms via alternative RNA splicing, namely skeletal muscle β -TM and fibroblast TM-1. The latter is also expressed in smooth muscle where it corresponds to smooth muscle β -TM. Skeletal muscle β -TM contains exons 7 and 10, whereas exons 6 and 11 are used in fibroblasts and smooth muscle. In order to study the properties of the alternatively spliced proteins, recombinant TMs derived from bacterial and insect cell expression systems were produced, including the normal β gene products, fibroblast TM-1 and β skeletal muscle TM, two carboxy-terminal chimeric TMs, TM-6/10 and TM-7/11, as well as a carboxyl-truncated version of each, TM-6/Cla and TM-7/Cla. The purified TM isoforms were used in actin filament association studies. The apparent TM association constants (K_a) were taken as the free concentration at half saturation and were found to be 6 μ M for β Sk TM, 8.5 for TM-6/10, 25 μ M for TM-1, and 30 μ M for TM-7/11. For the truncated TMs, the values determined were higher still but the binding was not carried out to full saturation. Isoforms were also produced using the baculovirus-insect cell system which produces proteins with an acetylated amino terminus as is normally found *in vivo*. This modification significantly enhanced the F-actin association of TM-1 but not the β skeletal TM or the other isoforms. Fibroblast TM-2 or TM-3, both products of the α gene, enhanced the affinity of TM-1 for F-actin, demonstrating different isoforms can act cooperatively on binding to actin. This effect was not detected with the other expressed β gene products. The presence of 83 kd nonmuscle caldesmon was found to enhance the binding of TM-1 for F-actin. This effect was dependent on the presence of both exons 6 and 11, as caldesmon had little effect on the other β gene products. Collectively, these results demonstrate TMs differ in their affinity for F-actin, which can be altered by other TMs or actin-binding proteins. The β tropomyosin isoforms were fluorescently-tagged and microinjected into cultured cells to study their *in vivo* localization where it was found that each of the full-length TMs bound to microfilaments but, at the light microscopy level, the isoforms were not differentially localized in these fibroblasts.

GENETIC AND MOLECULAR CHARACTERIZATION OF
SCRAMBLED; A LOCUS REQUIRED FOR MITOTIC ACTIN
ORGANIZATION AND FUNCTION.

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The *Drosophila scrambled* mutation prevents actin reorganization in response to the biochemical signals that trigger entry into mitosis, but does not affect interphase actin organization, cell cycle progression or the changes in microtubule and nuclear structure that normally accompany mitosis. The *scrambled* gene product thus defines a pathway leading from the cell cycle oscillator to the actin cytoskeleton. To gain insight into the biochemical properties of this pathway, we have initiated a genetic and molecular characterization of the *scrambled* locus. The original scrambled allele is associated with a P element transposon insertion. A series of six overlapping deficiencies and *in situ* hybridization studies place the *scrambled* gene in polytene chromosome region 42AB. Excision of the P element transposon that is associated with *scrambled* produced both revertants that are fertile and new alleles of the *scrambled* gene. These observations indicate that the transposon insertion is directly responsible for the mutant phenotype. Sequences flanking the P element have been isolated and were used to recover additional genomic clones from the *scrambled* region. These sequences are being used to identify candidate *scrambled* transcripts. Our progress toward identification of the *scrambled* gene will be reported.

DYNEIN REGULATORY MUTATIONS IN *CHLAMYDOMONAS*,
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To generate the flagellar waveform, the pattern of dynein activity must be highly regulated both around and along the length of the axoneme. Several lines of evidence have implicated the radial spoke-central pair (RS/CP) complex as a key control element, in particular because *Chlamydomonas* mutant strains that lack these structures are paralyzed (reviewed in 1). What is still unknown is the nature of the signals between the RS/CP complex and the dynein motors as well as the location of the domains within the dyneins that receive and respond to these signals. To address these questions, we have characterized a group of regulatory mutations that were obtained as extragenic suppressors of the paralyzed RS/CP defective phenotype (2-3). These suppressors are thought to modify components in the signaling pathway between the RS/CP complex and the dyneins. Our analysis has revealed regulatory elements in both the inner and outer dynein arms. One class of suppressor mutation (*sup-pf-1* alleles) produces specific modifications within the structural gene for the beta dynein heavy chain (DHC) of the outer dynein arm. These mutations are in-frame deletions in a region encoding a small but conserved alpha-helical coiled-coil domain. This domain appears to coordinate activity between the multiple dynein isoforms (2&4). A second class of mutation (*sup-pf-2*) alters the assembly of the outer dynein arm on specific doublet microtubules of the axoneme. This result suggests that an asymmetry in outer arm activity can compensate for defects in the RS/CP complex. Another class of mutation (*pf9-2*), which disrupts the assembly of the I1 inner arm dynein complex, implicates this isoform as a potential regulatory target (3). The last group of mutations (*sup-pf-3*, *sup-pf-4*, *pf2*, *pf3*) alter the assembly of a group of axonemal polypeptides known as the "dynein regulatory complex" or DRC (2&5). Analysis of these mutations suggest that the DRC components form a discrete, crescent-shaped structure in the inner arm region, where it is ideally positioned to mediate local signals, either mechanical, chemical, or both between the different dyneins (6-7). Because of the importance of the inner arms as regulatory targets, we have now used a PCR-based strategy to isolate nine members of the inner arm DHC gene family (see abstract by Myster et al., these proceedings).

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(3) Porter et al., 1992, J. Cell Biol. 118:1163 (4) Porter et al., 1994, J. Cell Biol.
126:1495 (5) Piperno et al., 1992, J. Cell Biol. 118:1455 (6) Mastronarde et al.,
1992, J. Cell Biol. 118:1145 (7) Gardner et al., 1994, J. Cell Biol., 127:1311.

**THE ROLE OF IFAP300 IN REGULATING INTERMEDIATE
FILAMENT INTERACTIONS WITH THE CELL SURFACE AND
OTHER CYTOSKELETAL SYSTEMS.**

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The intermediate filament (IF) system is organized into a continuous network which is located between the nuclear and cell surfaces. This cytoskeletal network appears to be maintained by numerous interactions including those between IF; IF and cell surface associated structures such as desmosomes and hemidesmosomes; and between IF and other cytoskeletal components. Very little is known about the molecular and structural basis of these various interactions, but it is obvious that IF associated proteins (IFAPs), such as IFAP300, must be involved. For example, morphological data shows that in epithelial cells IFAP300 plays a role in desmosome assembly as well as in the attachment of keratin IF to the most proximal plaque region of desmosomes. Biochemical analyses show that keratin IF bind to IFAP300 *in vitro*. These observations suggest that IFAP300 is involved in linking IF to the epithelial cell surface. In fibroblasts (which contain vimentin IF), immunofluorescence observations show that IFAP300 is associated with the entire IF network. Furthermore, IFAP300 binds to and co-pellets with vimentin IF assembled *in vitro*. In order to study the functional significance of the binding of IFAP300 to vimentin IF *in vivo*, we have carried out studies involving the microinjection of affinity purified IFAP300 antibodies. Within 30 minutes following microinjection, a dramatic reorganization of the IF network is seen which results in the formation of large juxtanuclear aggregates. These antibody injections are also found to have a significant effect on the organization of microtubules which appears to involve their depolymerization in the centrosomal region. These results indicate that IFAP300 may be involved in regulating the interactions between microtubules and IF in fibroblasts. Taken together these data suggest that IFAP300 plays a central role in regulating the supramolecular organization of IF, as well as in determining their interactions with other cytoskeletal elements and the cell surface. (Supported by GM36806).

THE ACTIN REGULATOR RhoB IS CENTRAL TO RAS-INDUCED CELL TRANSFORMATION AND TO THE MECHANISM OF A NEW CLASS OF ANTI-CANCER AGENTS THAT BLOCK MALIGNANT CELL GROWTH

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Farnesyl transferase inhibitors (FTIs) are a new class of anti-tumor agents that inhibit malignant growth of Ras-transformed cells *in vitro* and *in vivo* without affecting the growth or viability of normal cells. These drugs inhibit the activity of farnesyl transferase, the housekeeping enzyme responsible for the functionally required posttranslational farnesylation of a small set of cellular proteins, including the Ras oncoprotein. Our studies have revealed that a critical aspect of the FTI mechanism of action involves RhoB, a farnesylated small GTP-binding protein which has been implicated in cytoskeletal actin regulation.

It was observed previously that the biological mechanism of FTIs is complex and is poorly correlated with their inhibition of Ras farnesylation (1). The ability of FTIs to induce actin stress fibers in both normal and Ras-transformed cells (1) suggested that FTIs might inhibit malignant cell growth by interfering with cytoskeletal actin regulation. We speculated that RhoB may play a critical role because it regulates stress fibers and because as a farnesylated protein is in principle vulnerable to inactivation by FTIs. Therefore, we tested the hypothesis that RhoB was required for FTI activity and for Ras-dependent cell transformation.

A dominant inhibitory RhoB mutant (RhoB-N19) inhibited transformation by Ras but not by v-Raf, which acts downstream and independently of Ras. Activated RhoB mutants lacked significant cell transforming activity but augmented the activity of Ras. Rat1 cell lines stably expressing activated RhoB grew to higher saturation density and displayed reduced anchorage requirements for growth, supporting a role for RhoB in cell growth regulation. To determine if loss of RhoB was necessary for FTI action, we compared the response of Ras-transformed cell lines that contained normal farnesylated RhoB or RhoB constructs whose activity depended on N-terminal myristylation instead of C-terminal farnesylation (Myr-rhoB genes). Significantly, expression of Myr-rhoB made Ras-transformed cells resistant to FTI-induced growth inhibition and morphological reversion. We concluded that RhoB activity played a critical role in Ras-dependent cell transformation and in the mechanism by which FTIs inhibit malignant cell growth.

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GELSOLIN-RELATED PROTEINS OF INVERTEBRATE MUSCLE

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Gelsolin-related proteins occur in two principal types throughout the animal kingdom: either in the form of the six domain-proteins (~80kDa) represented by mammalian gelsolin or as the three domain-proteins (~40kDa) identified first in slime molds (severin, fragmin). Both types are structurally related, and the most characteristic property of all these proteins is the Ca-dependent actin filament severing.

Recently we have structurally and functionally characterized gelsolin-related proteins from annelid muscle (40kDa) and crustacean muscle (80kDa) (Bock et al, Eur J.Biochem. 225, 727-735, 1994; Giebing et al., Eur. J. Biochem. 225, 773-779, 1994; Lück et al., Biochem. J. 305, 767-775, 1995). Despite their strong sequence homology to mammalian gelsolin, individual properties of invertebrate proteins may be significantly different, i.e. the gelsolin isolated from crayfish tail muscle formed complexes with three actin molecules, and all three actins bound in a Ca-dependent and EGTA-reversible manner. Further differences to mammalian gelsolin were found with respect to the nucleation of actin polymerization and actin filament severing.

Muscles from species of different animal phylae showed differential cross-reactions in immunoblots with anti-fragmin, anti-lobster gelsolin and vertebrate gelsolin. While none of the invertebrate tissues cross-reacted strongly with antibodies against vertebrate gelsolins, ~40kDa proteins were detected in molluscan muscles (Scallop adductor), in primitive chordates (Branchiostoma) and in echinoderms with either anti-fragmin, anti-crustacean gelsolin or both, in addition to the proteins from annelid and crustacean muscle mentioned above. So far in none of the muscle samples a clear coexistence of 40 and 80kDa immunoreactive components could be demonstrated. The 40kDa protein detected in Scallop adductor muscle has now been isolated and functionally characterized as a Ca-dependent actin filament severing protein.

Regardless of whether the antibodies had identified a 40 kDa or 80 kDa type protein in the immunoblots and whether anti-fragmin or anti-crustacean gelsolin were used for immunofluorescence, characteristic staining patterns were observed in both longitudinal cryosections of cross-striated muscles and isolated myofibrils. These patterns varied with the degree of contraction but always revealed a localization throughout the I-Z-I regions of the sarcomers, the staining being coincident with the rh-phalloidin staining for filamentous actin. It was concluded from this pattern that a significant proportion of gelsolin-related proteins is associated with the thin filaments in the muscle cell.

CHARACTERIZATION OF CLIP-170, A CYTOPLASMIC LINKER PROTEIN Janet E. Rickard, Georgos Diamantopoulos and Thomas E. Kreis, Département de Biologie Cellulaire, Sciences III, Université de Genève, CH-1211 Genève 4

CLIP-170 is a microtubule-binding protein originally identified in HeLa cells which has been implicated in endosome-microtubule interactions (1). It has also been localized to desmosomal plaques in polarized epithelial cells (2), suggesting that it may function to link diverse cytoplasmic organelles to microtubules. In fibroblast-like cells, CLIP-170 accumulates at the microtubule plus ends (3), the site of polymer turnover. The interaction of CLIP-170 with microtubules is regulated by phosphorylation in vitro, and the phosphorylation of CLIP-170 in vivo is influenced by perturbation of the microtubule polymer state (4). These data suggest a relationship between the phosphorylation and microtubule-binding activity of CLIP-170 and microtubule dynamics, and are consistent with the hypothesis that CLIP-170 has a higher affinity for the stabilizing 'GTP-cap' conformation of tubulin. We are currently testing this model for targeting of CLIP-170 to microtubule plus ends. The interaction of CLIP-170 with microtubules polymerized with GTP or non-hydrolyzable analogues is being investigated and preliminary results indicate that CLIP-170 binds preferentially to GTP γ S microtubules in vitro, suggesting that it may indeed distinguish between different conformations of tubulin. To understand how the phosphorylation of CLIP-170 may be regulated by microtubules, we are trying to identify the phosphorylation sites on CLIP-170, with the eventual aim of identifying the specific kinase and phosphatase. Results obtained so far suggest that at least some of the in vivo phosphorylated residues are close to the two conserved microtubule-binding domains found in the N-terminal region of the protein, consistent with a role for this modification in regulating the affinity of CLIP-170 for microtubules. Localization of CLIP-170 to microtubule plus ends, combined with its ability to interact with other cytoplasmic structures, may provide a cellular mechanism for control of microtubule turnover as well as promoting the microtubule-organelle interactions essential for cytoplasmic organization and the regulation of membrane traffic.

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**LOCALIZATION OF THE F ACTIN BINDING PROTEIN ABP-240
IN DICTYOSTELIUM DISCOIDIEUM.** A. Ridsdale and J. Condeelis.

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Dictyostelium is unusual in having two members of the non-muscle filamin family (ABP-120 and ABP-240 kD) that are able to cross link actin filaments into gels with nearly orthogonal filament intersections. Mono-specific, affinity-purified polyclonal antibodies were used for immunofluorescence imaging of both ABP-120 and ABP-240 relative to F-actin using a confocal microscope. Starved cells, exposed to cAMP for one minute, as well as unstimulated and streaming starved cells were examined. In general, while ABP-120 has a localization pattern similar to that of actin, ABP-240 is much more widely distributed throughout the cell volume where the F-actin concentration is very low. Cells that have become spread in response to cAMP stimulation show some enrichment of ABP-240 in the apical regions of the cell. There is also some depletion of ABP-240 in the outer edge of hyaline lamellapodia. ABP-120 is not found in the central volume of the cell but largely in apical surface structures which are also rich in actin. It is especially enriched in apically forming pseudopodia which apparently constitute the leading edge of the cell. ABP-120 is also found associated with actin at the outer edge of hyaline lamellapodia. The non-overlapping distributions of these two proteins suggest very different, and perhaps complimentary functions. ABP-120 is always associated with actin enriched apical structures while ABP-240 may become transiently associated with the sub-cortical regions of nascent pseudopodia.

GENETIC AND BIOCHEMICAL ANALYSIS OF OVARIAN RING CANAL PROTEINS

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During *Drosophila* oogenesis, the oocyte develops from a cluster of 16 germline-derived cells that arise from four rounds of mitosis characterized by incomplete cytokinesis. The mitotic cleavage furrows are arrested and transformed into ring canals that provide a pathway into the oocyte for nurse cell cytoplasm flow. The ring canal has been shown to contain actin, at least one protein product from each of the *hu-li tai shao* (*hts*) and *kelch* genes, and at least one phosphotyrosine-containing protein. We analyzed ring canals by sucrose density gradient centrifugation and found that the 80 kD and 170 kD *kelch* proteins and the 60 kD *hts* proteins co-sediment with the ring canal complex. The 80 kD *kelch* protein contains a 120 amino acid BTB box near the amino-terminus and a 300 amino acid *kelch* repeat domain in the carboxy half of the protein. This repeat domain is conserved in a wide variety of proteins, including *Limulus* *scruin*. *Scruin* contains two sets of *kelch* repeats, each of which appears to be an actin-binding domain. A bacterially-produced *kelch* BTB domain forms a dimer in solution. This suggests that *kelch* might be a dimeric actin binding protein that may be capable of bundling actin filaments. We are expressing *kelch* ORF1 in baculovirus to obtain purified protein to test these hypotheses. Furthermore, we are testing germline-transformants carrying the wild type *kelch* cDNA, ORF1 only mutant cDNA, or full-length only mutant cDNA to determine which of the *kelch* proteins is necessary to rescue *kelch* mutants. We are also pursuing the cloning of a new female sterile mutant, *cheerio*, which maps to 89F and affects ring canal structure. Immunofluorescence experiments have shown that only the phosphotyrosine protein is present on *cheerio* mutant ring canals. Ring canals fail to grow to normal size and some deteriorate. This evidence suggests that the *cheerio* gene product is required early in ring canal biogenesis.

DIFFERENCES OF CYTOSKELETAL DYNAMICS IN CULTURED NEONATAL AND ADULT RAT CARDIOMYOCYTES

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Neonatal and adult rat cardiomyocytes in culture undergo a sequence of morphological changes in the presence of serum. Initially neonatal rat cardiomyocytes (NRC) are round and after attachment to the substrate the cells begin to spread and myofibrils emerge. Adult rat cardiomyocytes (ARC) however start from a rod-shaped cell, round up and most myofibrils degenerate. The cells then flatten, grow in size and myofibrillar assembly occurs. Microfilaments and microtubules can be influenced by addition of drugs. Cytochalasin D (CD) binds to the barbed end of the actin filaments, nocodazole (NO) inhibits the addition of tubulin to microtubules and both result in filament depolymerization. NRC treated with CD are able to attach to the substrate but are totally inhibited from spreading. However, even if the microtubules in NRC are destroyed with NO the cells are able to attach and to spread but not to the same extent as control cells. ARC treated with CD or NO are completely inhibited from spreading but remain attached to the substrate. The drug treatment does not inhibit protein synthesis neither in NRC nor in ARC. While in treated NRC myofibrils survive, in ARC a complete degeneration is observed and the accumulation of myofibrillar proteins is decreased. Cell spreading and myofibrillogenesis in NRC appears to be more dependent on functional microfilaments whereas in ARC spreading is dependent on functional microfilaments and microtubules as well. In addition, ARC, inhibited from spreading, appear to have a degradation system which seems not to be present in NRC.

MITOTIC FUNCTION OF KAR3, A MINUS END-DIRECTED MICROTUBULE MOTOR PROTEIN.

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KAR3 is a minus end-directed microtubule motor protein that is essential for karyogamy and important for mitosis in *S. cerevisiae*. During mitosis, *kar3*- mutants grow slowly, lose chromosomes at an elevated rate, and accumulate cells with bipolar spindles, consistent with a role in spindle elongation during anaphase (Cell 60:1029, 1990). Kar3p is phosphorylated in vegetative cells and the stoichiometry of phosphorylation is reduced in cells arrested in G₁ by α F (with P. Meluh). We mapped the phosphorylation sites to the N-terminal 57 amino acids by comparing tryptic peptide maps of ³²P-labeled Kar3p immunoprecipitated from a collection of *kar3* deletion mutants with maps of full length Kar3p. The tryptic phosphopeptides correlate to regions of the protein that contain four CDC28 kinase consensus sites. Furthermore, bacterially-expressed Kar3p is phosphorylated *in vitro* by cyclin-p34^{cdc2/CDC28} kinase at the same sites phosphorylated *in vivo*. We have begun systematic site-directed mutagenesis of all possible combinations of the four CDC28 consensus sites, and have assayed the point mutants for karyogamy and mitotic function. In preliminary experiments, substitution of threonine-7 and threonine-39 (*kar3-T7A T39A*) with alanine yields a recessive allele of *kar3* that is both defective for karyogamy and displays slow growth comparable to that of a *kar3* deletion (*kar3Δ*). Surprisingly, *kar3-T7A T39A* accumulates cells in mitosis with monopolar spindles, whereas *kar3Δ* arrests predominately with bipolar spindles. Thus, phosphorylation of Kar3p appears to be required for bipolar spindle assembly or maintenance. Saunders and Hoyt proposed that a bipolar spindle in *S. cerevisiae* is assembled in response to opposing forces from the two classes of microtubule motor proteins of opposite directionality (Cell 70:451, 1992). The redundant pair of putative plus-end directed motors, *CIN8* and *KIP1*, exert a net inward force that is countered by a net outward force from *KAR3*. In support of this model, the collapse of bipolar spindles observed in *cin8(ts) kip1Δ* strains is partially suppressed by *kar3Δ*. Considered in this context, the *kar3-T7A T39A* allele appears to be a hyperactive form, preventing bipolar spindle assembly in the presence of wildtype *CIN8* and *KIP1*. Thus, phosphorylation by cyclin-cdc28 kinase may inhibit Kar3p function until loss of kinase activity at anaphase, when dephosphorylated active Kar3p may function in chromosome segregation. We are currently testing this model by 1) attempting to suppress *kar3-T7A T39A* by overexpression of *CIN8* and *KIP1*, 2) comparing phosphorylation of Kar3p in metaphase to that in anaphase and 3) examining Kar3p phosphorylation in *cdc* mutants. We also are investigating a possible role of phosphorylation of Kar3p at threonine-7 in mitotic spindle feedback control.

**Remodelling of Cytoskeletal and Contractile Structures and
Changes in ANF Production under the Influence of IGF-I and bFGF
in Adult Rat Cardiomyocytes in Long-term Culture**

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Isolated adult rat cardiomyocytes follow a defined sequence of shape changes during culturing in the presence of fetal calf serum (Eppenberger ME et al. *Dev. Biol.* 130: 1-15. 1988). Rod-shaped cells round up, attach to the substratum and start to grow out in all directions until they form contacts with one another. Cells are hypertrophying amitotically. After 9 to 14 days, cell shapes include large round pseudopodia-like structures as well as polygonal forms with triangular extensions. Myofibrils were visualized in confocal microscopy with a monoclonal anti-myomesin antibody (Grove BK et al. *J. Cell. Biol.* 98: 518-524. 1984) in conjunction with staining F-actin by phalloidin-rhodamine. All cells contain usually a dense actin filament network (stress fibre-like structures) reaching out into the periphery. Myofibrils remain concentrated first in the perinuclear regions, and later grow slowly following the actin stress fibres into the periphery. Finally, myofibrils are filling the entire cell body in an almost superimposable pattern to the actin network. Deviations from this general pattern were observed:

With IGF-I (insulin-like growth factor), cells are of normal size. The actin network is well developed and myofibrils grow out fast into the periphery following the actin network. Very few cells are staining with a monoclonal antibody specific for alpha-smooth muscle actin (alpha-smA). In addition, ANF production is down-regulated. With bFGF (basic fibroblast growth factor), all cells get very large, often forming broom-like extension structures. Myofibrils remain restricted in the perinuclear regions and do not follow the cytoskeleton into the periphery. Almost all cells stain heavily for alpha-smA, and ANF production is up-regulated.

In all cases, the stress fibre-like structures of actin seem to serve as a scaffold for the out-growing myofibrils. However, the two structures are regulated differentially. Myofibrils are following the actin scaffold right into the periphery in control cells as well as in the presence of the differentiating growth factor IGF-I. In contrast, treatment with the differentiation-inhibiting FGF restricts the myofibrillar structures to the perinuclear regions. Control cells and all treated cells exhibit good contractile activity despite the morphological differences in myofibrillar distribution.

STRUCTURE / FUNCTION STUDIES ON *Dictyostelium* SEVERIN AND PROFILIN

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The three-dimensional structure of domain 2 of *Dictyostelium* severin in aqueous solution was determined by NMR spectroscopy¹. Severin is a Ca^{2+} activated actin-binding protein that severs F-actin, nucleates actin assembly, and caps the fast-growing ends of actin filaments^{2,3}. Domain 2 consists of a central five-stranded β -sheet sandwiched between a four-turn α -helix and, on the other face, a roughly perpendicular two-turn α -helix. There are two distinct binding sites for Ca^{2+} located near the N- and C-termini of the long helix. Conserved residues contribute to the apolar core, so that the overall fold of the protein is similar to those of segment 1 of gelsolin / villin and of profilin.

To study in vivo functions of the ubiquitous actin-binding protein profilin we generated by anti-sense and gene disruption techniques *Dictyostelium* mutants that lack one or both of the profilin isoforms⁴. Whereas the single mutants showed an essentially unchanged phenotype as compared to wild-type cells, the behaviour of the double mutant was drastically altered: Motility was significantly reduced, single cells were up to 10-times larger than wild-type cells and showed a broad rim of F-actin below the plasma membrane, the F-actin concentration was increased by about 60-70%, and development was blocked prior to fruiting body formation. Furthermore, double mutants could not be grown in shaking culture under normal conditions reflecting an impaired cytokinesis. The aberrant phenotype could be rescued by re-introducing a functional profilin I or profilin II gene. The data in this study suggest that profilin functions in *Dictyostelium* amoebae primarily as an actin sequestering protein. Using a newly constructed expression vector which allows selection for hygromycin resistant colonies, we currently try to rescue the aberrant phenotype of the pI/II-minus mutant by using point-mutated profilin and profilin from other organisms.

¹ Schnuchel et al., 1995, *J. Mol. Biol.* 247: in press; ² Eichinger et al., 1991, *J. Cell Biol.* 112:665-676; ³ Eichinger and Schleicher, 1992, *Biochemistry* 31:4779-4787; ⁴ Haugwitz et al., 1994, *Cell* 79:303-314.

α -CARDIAC ACTIN CAN SUBSTITUTE FOR INDIRECT FLIGHT MUSCLE ACTIN IN TRANSGENIC *DROSOPHILA*

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Actin is not only involved in the generation of movement and force, but also plays a role in the structure and dynamics of the cytoskeleton. The expression of multiple, closely related isoforms which is tissue-specific and developmentally regulated, suggests that different functions exist for different isoforms.

We have used transgenic *Drosophila* to study the effect of ectopically expressed human α -cardiac actin on the organization and function of indirect flight muscles (IFM). Of the six actin isoforms in *Drosophila*, the Act88F, is exclusively expressed in IFM. Surprisingly, all *Drosophila* actins, including Act88F, are more similar in sequence to vertebrate non-muscle actins than to skeletal α -actins. The vertebrate α -cardiac actin differs by 28 residues from Act88F, a large divergence for the highly conserved actin family. To explore to what extent sequence differences determine the structure and function of isoforms, we substituted Act88F with human α -cardiac actin by P-element mediated germ line transformation of KM88 recipient flies that are null mutants for Act88F. To distinguish the transgenic α -cardiac actin from endogenous actins, the carboxy-terminus was tagged with an 11-amino acid epitope from vesicular stomatitis virus G protein. Transformants that overexpressed the transgene were flightless and sarcomeric organization was not evident. However, in transgenic lines that expressed the tagged α -cardiac actin at levels comparable to Act88F in wild-type flies, IFM function and architecture appeared entirely normal. IFM fixed *in situ* displayed well-ordered myofibrils with uniform thin filament length and normal myotendon junctions. The phenotype of transgenic IFM suggests that in spite of the considerable sequence divergence between vertebrate α -cardiac and *Drosophila* Act88F, these two isoforms appear structurally and functionally equivalent.

To learn more about the significance of sequence differences, we are currently investigating the effect a mutated actin isoform and an actin related protein which has less sequence homology than the different actin isoforms, on the function and organization of IFM.

INTERACTION OF PEROXISOMES WITH MICROTUBULES IN VIVO AND IN VITRO. (M. Schrader, J.K. Burkhardt¹, E. Baumgart, G. Lüers, A. Völkl, and H.D. Fahimi) Institute for Anatomy and Cell Biology II, University of Heidelberg and European Molecular Biology Laboratory¹, Heidelberg, Germany.

We have investigated the role of the cytoskeleton in determining the shape and intracellular distribution of peroxisomes. For our studies we used the well differentiated human hepatoblastoma cell line HepG2 exhibiting a large number of morphologically heterogeneous peroxisomes (Schrader et al., 1994, Eur. J. Cell Biol. 64, 281-294). A variety of microtubule-depolymerizing drugs (Colcemid, Nocodazole, Vinblastine) induced significant increases in frequency of tubular peroxisomes and led to the formation of peroxisomal clusters. Taxol, a microtubule stabilizing drug, affected neither the shape nor the intracellular distribution of this organelles. Furthermore, the association of spherical peroxisomes with microtubules was clearly visualized by confocal laser scanning microscopy using antibodies to catalase, a peroxisomal marker enzyme, and to tubulin for double immunofluorescence. An in vitro binding assay was established using highly purified rat liver peroxisomes isolated by Metrizamide density gradient centrifugation and Taxol-stabilized microtubules from bovine brain. Video-enhanced microscopy revealed strong binding of purified peroxisomes to microtubules. Binding was abolished by pretreatment of peroxisomes with protease or KCl. Whereas the binding of KCl-treated peroxisomes could be restored by the addition of cytosol, untreated control peroxisomes bound to microtubules equally well in the presence or absence of cytosol. These data show for the first time specific binding of peroxisomes to microtubules suggesting that microtubules play an important role in determining the shape and intracellular distribution of peroxisomes. Supported by SFB 352 and LFSP of the State of Baden-Württemberg, Germany.

STRUCTURAL STUDIES ON TAU PROTEIN AND PAIRED HELICAL FILAMENTS BY X-RAY SCATTERING, SPECTROSCOPY, AND ELECTRON MICROSCOPY.

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In order to understand the principles by which paired helical filaments (PHFs) are formed from tau protein in Alzheimer's disease it is necessary to investigate the structure of both the monomeric and polymeric protein. The amyloid (A β) can serve as a paradigm: The peptide assumes a beta conformation in solution which favors aggregation, as judged by circular dichroism (CD) [1], and the assembled amyloid fiber has predominantly a cross-beta conformation, as judged by X-ray fiber diffraction [2]. In the case of tau, a similar cross-beta structure has been proposed for paired helical filaments [3], yet monomeric tau showed very little beta-conformation by CD; the same is true for tau constructs that can self-assemble into PHF-like fibers in vitro [4]. In order to resolve this paradox we have studied tau protein by CD, X-ray scattering and electron microscopy. Similarly, we studied PHFs prepared from Alzheimer brain tissue by X-ray fiber diffraction, electron microscopy and Fourier transform infrared spectroscopy (FTIR). The results show that both tau and PHFs have very little secondary structure [5]. Tau does not even have a compact folding but rather behaves like a denatured protein in solution [5].

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SIDE-ON MICROSCOPY: A NEW PERSPECTIVE SHOWS CELL SHAPE GENERATION REQUIRES MYOSIN II.

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Myosin II, together with filamentous actin, forms the basis of contractility in muscle and non-muscle cells. Observation of *D. discoideum* amoebae has shown that cells lacking functional myosin II remain viable and motile, although some defects in morphology and translocation rate are detected. These studies used 2-dimensional imaging techniques allowing observation only within the plane of the cell substrate and thus 3-dimensional cell behavior has not been examined. We have developed methods allowing observation of cells from the side (side-on), normal to the substrate plane. Time-lapse side-on observation reveals that axenic wild-type cells undergo dramatic 3-dimensional movement and shape change, extending away from substrate attachment sites (vertically) at rates comparable to that of cell translocation. Such extensions are further decorated with protrusions which may represent pseudopods seen using conventional imaging methods. Similar analysis shows that myosin null mutant (*mhcA*-) cells cannot extend vertically but form small protrusions in all directions and spread laterally upon the cell substratum. Additionally, wild-type cells suspended in medium continue to exhibit elaborate cell shapes, while *mhcA*- cells in suspension are spherical, with the exception of small protrusions. Images of fixed cells stained with fluorescent phalloidin obtained using side-on confocal fluorescence microscopy or by volume rendering of optical sections reveals that small protrusions generated by wild-type and *mhcA*- cells contain dense F-actin networks. However, vertical extensions formed by wild-type cells are not F-actin enriched, do not exclude organelles, and appear to be extensions of the general cell cytoplasm. We conclude that vertical cell extension requires myosin II and is mechanistically distinct from pseudopod formation and cell spreading. *D. discoideum* amoebae undergo aggregation and then formation of slugs and fruiting bodies when starved. Previous analysis has shown that starved *mhcA*- cells aggregate but fail to undergo further morphogenesis. We suggest that morphogenesis requires cells to generate 3-dimensional shapes within the confines of a multicellular mass, and that *mhcA*- cells are blocked in development because they cannot generate independent cell shape but can only conform to the shape of their substrate.

The immunosuppressive atypical sigma ligand SR 31747 blocks cell proliferation by inhibiting a steroid isomerase in *Saccharomyces cerevisiae*

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SR 31747 [(2*N*-cyclohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride] is an atypical sigma ligand which blocks the proliferation of lymphocytes, likely affecting a late event during the S phase. As the mechanisms of action of various immunosuppressive drugs, such as cyclosporin A, FK506 and rapamycin, are strikingly conserved from human T cells to yeast, we have studied the effect of SR 31747 on the yeast *Saccharomyces cerevisiae*. Here we show that yeast is sensitive to SR 31747 and that the products of two genes, namely *SKK1* and *SKN1*, are necessary to mediate the SR 31747-induced growth arrest. The overexpression of either of these genes confers hypersensitivity to SR 31747, whereas *skk1* or *skn1* gene disruptants are resistant to this drug. The *skn1-1* allele (Ladeveze *et al.*, *Lipids*, 28, 907-912) is resistant to various sterol biosynthesis inhibitors. Sterol analyses indicate that sterol isomerase is impaired in SR 31747-treated cells. Overexpressing *ERG2*, the sterol isomerase-encoding gene, confers resistance to SR 31747 in wild-type cells. The *ERG2* gene disruption is lethal, except in *skn1* or *skk1* mutants. We conclude that SR 31747 blocks the sterol biosynthesis pathway at the sterol isomerase step, in wild type cells, and in the *skk1* and *skn1* mutants. Microscopic observations and FACS analysis suggest a SR 31747-induced defect in cytokinesis. The role of the *SKK1* and *SKN1* gene products in mediating the SR31747 inhibitory effect will be discussed.

CYTOSKELETAL ROLE IN mRNA LOCALIZATION.

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We have investigated the mechanism by which actin mRNA can translocate to the leading edge of motile fibroblasts. This location of β -actin mRNA results in actin protein synthesis in the region of the cell actively involved in actin polymerization which drives motility. The fibroblast is a cell differentiated for directed motility in response to a chemo tactic gradient. The physiological relevance of mRNA localization is illustrated by the rapid localization of actin mRNA in response to chemotaxis. Serum stimulation or growth factors initiate a relocation of actin mRNA which was not localized in the cytoplasm of serum starved cells. This movement of β -actin mRNA to the leading edge takes only a few minutes and can be inhibited by drugs which interfere with phosphorylation of proteins. Herbimycin, a potent inhibitor of protein kinase C, prevents the localization in response to stimuli. The interaction of mRNA with the cytoskeleton appears to be through actin filaments. Electron microscopy combined with in situ hybridization shows mRNA, indicated by poly(A) hybridization, to be associated through actin filament intersections. This represents the anchoring site of mRNAs and colocalizes with actin binding proteins such as filamin (ABP280) and the elongation factor 1α . This intersection may represent a compartment of mRNA utilization, and perhaps a spatial component to mRNA translational control. In neurons, mRNA is transported down the axon and appears to be in close association with microtubules. At present, work is directed to isolating the sequences and the proteins associating with the actin filament network.

THE REGULATION OF MYOSIN II LIGHT CHAIN PHOSPHORYLATION IN DICTYOSTELIUM

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Myosin II activity in nonmuscle cells is increased by phosphorylation of the regulatory light chain by myosin light chain kinase (MLCK). Our molecular genetic analysis indicates that in *Dictyostelium* there are at least two MLCKs, and that one of these (MLCK-A) facilitates cytokinesis. Unlike muscle MLCKs, MLCK-A is not directly regulated by Ca^{2+} /calmodulin, but it is under cellular control. MLCK-A can be activated by treatment of cells with the lectin conA, which results in the rapid phosphorylation of essentially all of the regulatory light chain. cGMP activates MLCK-A in crude lysates, and is thus a candidate for the intracellular signal mediating conA activation. ConA treatment also results in the phosphorylation of MLCK-A. *In vitro*, MLCK-A autophosphorylates on threonine-289, which increases its activity. However, fully autophosphorylated MLCK-A still has only about 1% of the activity of MLCK-A in lysates from activated cells, indicating that while autophosphorylation probably plays a role in MLCK-A regulation, there is an additional mechanism for fully activating MLCK-A. A mutant MLCK-A with an alanine at the autophosphorylation site (T289A), has been expressed in MLCK-A⁻ *Dictyostelium*. T289A cells phosphorylate regulatory light chain in response to conA. In addition, the T289A kinase is phosphorylated in response to conA, indicating the presence of an additional phosphorylation site(s) on MLCK-A. These results, coupled with sequence comparisons with other kinases, have led us to propose that MLCK-A is activated by phosphorylation of threonine-166 in the "activation-loop" - a region for activating phosphorylations in other protein kinases. Experiments are currently underway to test this hypothesis.

**TIAM-1, AN INVASION-INDUCING GENE THAT ENCODES A
GDP-GTP EXCHANGER FOR Rho-LIKE GTPases**

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Michiels and John Collard**

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Tiam-1 has been identified as an invasion-inducing gene in T-lymphoma cells, by proviral insertion mutagenesis. Invasiveness, as assessed on a monolayer of fibroblasts, was induced by truncation and/or overexpression of Tiam-1. The full length Tiam-1 protein contains two Pleckstrin-homologous domains and a DH-domain. The latter domain has significant sequence similarity to the GDP-GTP exchange domain in the DBL proto-oncogene product, that has been shown to activate small GTP-binding proteins of the Rho family. Rho-like proteins, like RhoA and Rac1, have been implicated in signal transduction towards cytoskeletal components.

Several DH-domain containing genes have been identified as transforming genes in NIH3T3 cells. Therefore, different Tiam-1 constructs were transfected into NIH3T3 cells. Depending on the construct used, Tiam-1 induces morphological transformation and oncogenic potential in 3T3 cells. Immunofluorescence staining showed that the actin cytoskeleton was profoundly affected in cells expressing an N-terminally truncated Tiam-1 protein. Similar to constitutively active V12 Rac1, Tiam-1 induces formation of membrane ruffles in fibroblasts, suggesting that Tiam-1 activates Rac1. In addition, cell motility was affected, as assessed by phagokinesis- and woundhealing assays.

Both in T-lymphoma cells and in 3T3 transfectants we are currently characterising the Tiam-1 products with respect to turnover rate, phosphorylation and cellular localisation. (Supported by the Dutch Cancer Society).

IDENTIFICATION OF NEW MICROTUBULE BINDING PROTEINS IN *Dictyostelium discoideum*.

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Cells of the slime mold *Dictyostelium discoideum* are highly motile and rapidly change shape and polarity in response to external signals. The actin cytoskeleton of the amoeba is composed of a plethora of actin binding proteins (ABPs), that crosslink, bundle or sever actin filaments as well as proteins that sequester actin monomers or nucleate the polymerization of actin filaments. On the other hand the microtubule (MT) system of *Dictyostelium discoideum* is poorly defined, yet it should also be a dynamic and highly regulated structural assembly.

Recently we initiated a project to characterize components of the microtubule system. Our main approach has been to generate monoclonal antibodies in mice immunized with a microtubule stabilized cytoskeleton, prepared by extracting HG403 cells with 1% Triton in the presence of 20 μ M taxol and 0.5% glutaraldehyde. Western blot analysis confirmed the presence of tubulin in the immunogen. Fusions were screened by immunofluorescence. Five MT staining antibodies were generated by this method as well as a larger number of antibodies that gave an actin associated immunofluorescence staining. Of the five MT antibodies we have shown one reacts with α -tubulin whereas the other four probably label microtubule associated proteins (MAPs); they also crossreact with mammalian proteins.

mAb 212-174-1 (IgM) specifically labels MT at high antibody titer whereas at a lower titer a vesicular staining also is observed. This antibody does not recognize tubulin or any known motor protein from *Dictyostelium*. Screening of a *Dictyostelium* λ gt11-cDNA-library led to the identification of a protein of about 120 kDa. Surprisingly the sequence of this protein (1.5 kb to date) bears a 20% identity to members of the gelsolin family of ABPs. The predicted message size based on Northern blot analysis is 4.1 kb. Preliminary results show the protein is developmentally regulated reaching maximum levels after 3 hours of starvation.

More sequence data is being acquired to answer the question of whether the isolated ABP clone was found by serendipity, or whether the protein indeed acts as a crosslinker between actin filaments and microtubules. If this is the case then, since the immunofluorescence data does not reveal an actin like staining, we might expect the remaining sequence contains a microtubule binding motif that is recognized by the antibody.

EXPRESSION IN E. COLI AND PHOSPHORYLATION WITH CAMP DEPENDENT PROTEIN KINASE OF THE N-TERMINAL (160 KD) REGION OF HUMAN PLATELET ACTIN BINDING PROTEIN. Alfred Stracher, David Jay and Darryl Bing, Dept. of Biochemistry, SUNY Health Science Center, Brooklyn, USA.

Actin-binding protein, (ABP-280, nonmuscle filamin) is an essential component of the cytoskeleton where it promotes rearrangement of actin filaments in response to a variety of physiological and exogenous stimuli. Previously, we have postulated that a dynamic phosphorylation-dephosphorylation process may modulate the interaction of ABP with other cytoskeletal components. Indeed, ABP as isolated from platelets, already existed as a phosphoprotein that could not be phosphorylated by PKC but which retained its ability to organize F-actin into a highly cross-linked structure. Phosphorylation may also be a regulatory element in the determination of molecular stability. Thus, phosphorylation with PKA protected ABP from proteolytic degradation by calpain both, in vitro and in situ. In this case the phosphorylation site resided in the 90 kD carboxy-terminal fragment, the smaller of the two fragments produced by calpain cleavage. Analysis of the predicted amino acid sequence revealed multiple potential phosphorylation sites: three PKA sites, thirty three PKC sites, thirty casein kinase II sites and even one tyrosine kinase site. In order to gain further insight into the functional implications of phosphorylation of ABP we decided to express different parts of the molecule in a prokaryotic system because it lacked the phosphorylation machinery that characterizes eukaryotic cells. The N-terminal region of human platelet actin-binding protein was subcloned and expressed in the pT 7-7/*E. coli* BL 21 (DE 3) system. This peptide was efficiently expressed in *E. coli* as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by western analysis using a monoclonal antibody raised against this part of the molecule. As predicted by the amino acid sequence of this truncated protein (residues 21-1569), corresponding to 164 KD and containing the actin-binding domain near the amino-terminus, ABP could be phosphorylated by cAMP-dependent protein kinase unmasking a phosphorylation site which is not apparent in the native protein.

MOVEMENT OF SMOOTH ER ON ACTIN FILAMENTS IN THE SQUID GIANT AXON. J.S. Tabb^{1,3}, S.A. Kuznetsov^{2,3}, D.G. Weiss^{2,3} and G.M. Langford^{1,3}. ¹Department of Biological Sciences, Dartmouth College, Hanover, NH 09377, ²Fachbereich Biologie, Tierphysiologie, Universitat Rostock, Federal Republic of Germany, ³Marine Biological Laboratory, Woods Hole, MA 02543.

Several types of organelles, ranging in size from ~50 nm (size of synaptic vesicles) to > 1 μ m (size of mitochondria), have been shown to move on actin filaments in the squid giant axon (Kuznetsov et al., 1992, *Nature* 365:722-725), but the identity of these organelles has not yet been determined. We have begun to address this problem by studying the actin-dependent movement of one class of axoplasmic organelles identified as tubulovesicular organelles (TVOs). These organelles are likely to be either elements of smooth ER, tubular endosomes or prelysosomes, the former essential for Ca sequestration in the axon, and the latter two for synaptogenesis. When examined by AVEC-DIC microscopy, the vesicular domains within these tubulovesicular organelles were observed to move along actin filaments, but their movement was limited to short excursions because the tubular domains restricted the distance the vesicles traveled. The average velocity of these vesicular domains was 0.7 ± 0.4 μ m/sec (n=38), the same velocity that was measured for large round-shaped organelles (>800 nm) (Kuznetsov et al., 1994, *Cell Motil. Cytoskeleton*, 28:231-242). We obtained an enriched fraction of unfragmented TVOs by preparing axoplasmic ghosts. To prepare axoplasmic ghosts, extruded axoplasm was diluted into excess physiological buffer, which removed the diffusable cytosolic proteins, small organelles, and dynamically unstable cytoskeletal elements such as actin filaments and microtubules from the axoplasm. By AVEC-DIC microscopy, the TVOs within the ghosts were comprised of clusters of vesicles connected by tubular membranes of uniform diameter. The tubular components appeared as either long and stretched or relaxed and wavy. Electron microscopy showed that the vesicular domains varied from about 100-200 nm in diameter, and the tubules measured about 20-25 nm across. No membrane organelles other than TVOs were observed. TVOs stained with DiOC₆ became highly fluorescent. Both the vesicular domains and the tubular membranes were brightly stained. These data suggest that TVOs are elements of smooth ER rather than endosomes. To determine whether TVOs also sequester Ca, a major function of smooth ER, TVOs were incubated with Fluo-3, a Ca-sensitive fluorescent dye. AVEC-DIC and fluorescent images of the same TVOs show that only the vesicular domains, and not the tubular membranes were fluorescent. The presence of a fluorescent signal in the vesicular domains suggests that TVOs function in Ca sequestration. The observation that TVOs are labeled by DiOC₆ and by the Ca-sensitive dye, Fluo-3, strongly suggests that TVOs are related to smooth ER. The actin-dependent motility of TVOs provides evidence that the movement of smooth ER in the squid giant axon is driven by myosin-like motors. Future experiments using TVOs from axoplasmic ghosts and purified actin filaments will be used to directly demonstrate that the organelles can move along actin filaments. Supported by NIH Grant GM48438.

EFFECTS OF SMOOTH MUSCLE CALPONIN ON ACTIN ASSEMBLY.

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The effects of recombinant non-phosphorylated chicken gizzard alpha calponin on the kinetics of actin polymerization and the structure of the resulting actin filaments were studied by a combination of fluorescence, dynamic light scattering, polarized absorbance and electron microscopic assays. Substoichiometric amounts of calponin diminish the fluorescence increase associated with polymerization of pyrene-labeled actin but do not prevent actin polymerization. The suppression of fluorescence may be due in part to formation of large bundles of actin filaments whose average size depends on calponin concentration, ionic strength and adenine nucleotide concentration. Loose filament bundles with diameters of several hundred nm form in low ionic strength solutions. Millimolar concentrations of ATP disperse such bundles to single filaments or smaller aggregates. At higher ionic strength (150 mM KCl), filament bundles are smaller, but the bulk of filaments still assemble into lateral aggregates whose size depends on calponin concentration. These results show that calponin has effects on both the polymerization of actin and the supermolecular organization of actin filaments that could relate to its function as a regulator of contraction. Preliminary data also suggest more efficient bundling of alpha-smooth muscle actin (provided by C. Chaponnier, University of Geneva) than of alpha-skeletal actin. (supported by AR38910 to PAJ and PO1-AR41637 to TT).

TROPOMYOSIN ISOFORM EXPRESSION AND LOCALIZATION IN DIFFERENTIATING EPITHELIAL CELLS ((C. Temm-Grove¹, A. Watakabe¹, B.M. Jockusch² and D.M. Helfman¹)) ¹Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724; ²Cell Biology, Institute of Zoology, Technical University, 38092 Braunschweig, FRG.

Vertebrates contain four genes that express at least 16 distinct isoforms of tropomyosin (TMs), which are characteristic of specific cell types. We have been studying the expression and function of TM isoform diversity in various kinds of brush border (BB)-type epithelia (LLC-PK₁, Caco-2 and primary intestinal epithelium) and non-BB epithelia (NRK-52E) or fibroblasts (Ref-52). Western blot analyses were used to identify the isoforms in specific cell types. Primary BB cells and LLC-PK₁ cells were found to express the same isoforms of TM (TM-1, TM-2, TM-3, TM-5, TM-5a, TM-5b, TM-6), whereas the other cell lines exhibited some differences in the patterns of TMs expressed. Caco-2 cells do not express TM-1 and relatively low amounts TM-2, which is a characteristic of some transformed cell lines. Ref-52 and NRK-52E expressed TM-5a, and not TM-5b, whereas LLC-PK₁, Caco-2 and primary brush border cells all express preferentially TM-5b. Furthermore, TM-5b is regulated during differentiation, since LLC-PK₁ cells only express it after reaching confluence. Indirect immunofluorescence studies using a monoclonal antibody to HMW isoforms (TMs 1, 2, 3, 6) showed staining only of the stress fibers, not the adhesion belt. Conversely a polyclonal antibody recognizing LMW isoforms (TMs 5a, 5b, 5) stained only the adhesion belt. Using epitope-tagged constructs, immuno-fluorescence analysis with antibodies directed against the tag, revealed that high molecular weight TMs (TM-1, TM-2, TM-3, and TM- α sm) were localized to stress fibers, but not adhesion belts, whereas the low molecular weight TMs (TM-5, TM-5a, TM-5b) were found in both stress fibers and adhesion belts. Collectively these results demonstrate that epithelial cells express a complex pattern of TM isoforms, which exhibit differential localization within the cells and different patterns of expression depending on the origin of the epithelia.

DYNAMICS OF ACTIN MEASURED BY FLUORESCENCE CORRELATION MICROSCOPY (FCM).

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FCM collects information only from specific, fluorescently labelled, particles. The correlation functions obtained define not only the mobilities of the labelled particles (in terms of the average translational diffusion coefficient, D_T) but also the average number density ($\langle N \rangle$) of those particles within a defined volume. The sample volume is approximately $1 \mu\text{m}^3$ in the system we have developed.

We have used FCM to follow changes in $\langle N \rangle$ and D_T for both G-actin and actin polymers in free solution. G-actin concentration was determined by an indirect assay which actually measures the activity of known quantities of DNase1 (Huang *et al.* 1993). FCM measures the rate at which the enzyme generates DNA fragments (i.e $d\langle N \rangle/dt$) from labelled plasmid DNA in the presence of varying amounts of G-actin. With this method G-actin concentrations were assayed down to 100 pM. In such experiments the K_m of DNase1 for G-actin was $1.03 \times 10^9 \text{ M}^{-1}$ (SEM $\pm 10\%$), in good agreement with values obtained by other means. The polymerisation of actin in the presence of gelsolin was followed using BODIPY-phalloidin as a label. FCM was used to measure changing D_T values of actin filaments to quantify both the rate and extent of polymerisation from gelsolin-actin nucleation complexes. Mean equilibrium lengths of filaments were calculated from D_T values using diffusion equations for rigid rods (Broersma, 1981). Calculated lengths of actin filaments were, as expected, inversely proportional to molar ratios of gelsolin:actin over the range 1:50 to 1:100. FCM can measure D_T in the range 5×10^{-7} to $1.5 \times 10^{-9} \text{ cm}^2 \cdot \text{s}^{-1}$ corresponding approximately to filaments from 1 to 500 actin units in length.

FCM can detect green fluorescent protein (GFP). The availability of the cDNA for this unique fluorophore (Prasher *et al.*, 1992) will allow *in situ* expression of products such as actin, each molecule labelled specifically with an intrinsic fluorescent marker. FCM has the potential to quantify the dynamics of any cellular event which involves changes in $\langle N \rangle$ or D_T . GFP now makes it possible to realise this potential.

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ACTIN FILAMENTS IN THE RING CANALS OF *DROSOPHILA* OOCYTES.

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Connecting nurse cells to the developing oocyte are 15 intercellular bridges or ring canals. The canals increase from 0.5 μm in diameter at stage 2 to 10 μm in diameter at stage 11. From thin sections cut horizontally as you would a bagel, we show that there is a layer of circumferentially oriented actin filaments attached to the plasma membrane at the periphery of each canal. By decoration with subfragment 1 of myosin we find actin filaments in the ring of mixed polarities like the "contractile ring" formed during cytokinesis. From vertical sections through the canal the actin filaments appears as dense dots. At stage 2 there are 80 actin filaments in the ring and by stage 9 there are 800. Taking into account the diameter, this indicates that there is 120 μm of actin filaments/canal at stage 2 and 24,000 μm at stages 9-10. The density of actin filaments (number of actin filaments/ μm) remains unchanged during development at 3500/ μm^2 . From stage 2 to stage 5 the ring of actin filaments increases in thickness and in height. From stage 5 to stage 11 the thickness remains constant but the height increases from 0.5 μm at stage 5 to 2.1 μm at stage 9. At stage 5 the ring of actin appears homogeneous. As development proceeds and the height increases the actin filaments appear as parallel, circumferentially oriented bundles. In the null mutant, *kelch*, where a gene which codes for a protein located in the ring canal which is homologous to *scrib*, an actin binding protein (Way et al., 1995; JCB 128:51) the height of the ring at stage 9 is only 0.9 μm or 1/2 that of the wild type. Concomitantly the bundles of actin filaments extend from the ring proper into the lumen of the canal partly occluding the lumen. From these observations we speculate how a non-contractile ring increases in diameter, height, width and number of actin filaments. This must occur by the assembly of filaments and their interconnections to form the ring seen at stage 5 followed by the formation new bundles. These bundles form new rings like a series of washers lying on top of each other. The *kelch* gene product must function to hold these bundles together and to the plasma membrane.

DIRECT MEASUREMENT OF MICROTUBULE FLEXURAL RIGIDITY WITH THE LASER TRAP.

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Microtubules play pivotal structural roles throughout the cell cycle. As a component of the cell cytoskeleton, microtubules help maintain cell morphology and serve as a fibrous network for vesicular transport during interphase; during mitosis, microtubules comprise the spindle and are required to segregate chromosomes. Attempts have been made by several groups to estimate the stiffness or flexural rigidity of single microtubules *in vitro*. While there are general agreements in the estimated values of microtubule rigidity, all the estimated values were derived indirectly from the shapes of microtubules due to thermal fluctuation or hydrodynamic flow. We present here a direct measurement of microtubule flexural rigidity using a laser trap. The laser trap was calibrated by first trapping a 0.2 μm polystyrene bead, then the medium surrounding the bead was induced to move in a saw-tooth wave motion with known frequency and amplitude, and thus exerting a drag force on the bead. The drag force at the instant the bead escapes from the laser trap equals the force produced by the laser trap itself. To measure microtubule rigidity with the calibrated laser trap, purified tubulins were allowed to nucleate and polymerize into microtubules from axonemes adhered to the coverslips of a flow chamber. Beads coated with kinesin motors were introduced and allowed to bind tightly to the microtubule lattice. The laser trap at a high force setting was then used to trap the beads and bend the microtubules to a known geometry, typically parabolic-shaped. Then the force of the laser trap was slowly decreased until the bent microtubule stiffness overcame the trap and the microtubules sprang back to its original position. The experiment was recorded using VE-DIC microscopy.

Less than 1 pN force was sufficient to bend a 10 μm long microtubule to a 1 μm radius of curvature. The flexural rigidity of a microtubule was determined from the known laser trap force exerted and the geometry of the bent microtubule. We measured flexural rigidity values of $\sim 1.75 \times 10^{-23} \text{ Nm}^2$ for microtubules and $\sim 0.25 \times 10^{-23} \text{ Nm}^2$ for taxol-stabilized microtubules. We are attempting to measure the rigidity of microtubules under physiological conditions with *Xenopus* extracts.

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AN ANTIBODY DIRECTED AGAINST TRANSGLUTAMINASE ASSOCIATES
WITH VIMENTIN INTERMEDIATE FILAMENTS IN PRIMARY DERMAL
FIBROBLASTS.

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Detailed subcellular localization of TGase has not been described. A mouse monoclonal antibody G92.1.2 directed against guinea pig liver TGase recognizes a protein present in primary mouse dermal fibroblasts. When these fibroblasts are fixed and stained for indirect immunofluorescence with this antibody, a filamentous pattern bearing remarkable similarity to vimentin intermediate filament (IF) networks is seen. Double-label immunofluorescence reveals that the protein reacting with the TGase antibody co-localizes precisely with vimentin IF. Furthermore, this protein appears to co-localize with vimentin IF throughout the spreading and shape formation processes following trypsinization and replating of primary mouse fibroblasts. When these fibroblast cultures are treated with colchicine, which induces a perinuclear collapse of IF networks, the protein reacting with the TGase antibody remains co-localized with vimentin IF. This association with vimentin IF is retained when cells are extracted with non-ionic detergent in a high salt containing buffer. Western blots of the insoluble fraction following this extraction method show that the antibody directed against TGase recognizes a 280 kD protein in these preparations. The Western blots also show that the TGase antibody does not cross react with vimentin. Microinjection of monoclonal TGase antibody G92.1.2 into mouse dermal fibroblasts results in a collapse of the vimentin IF network. The results therefore suggest that not only is the TGase-like protein closely associated with the vimentin IF network of primary mouse dermal fibroblasts, but that it may also function in these cells as a cross-bridging protein, stabilizing the IF network. Supported by grants to R. Goldman (GM36806) and L. Lorand (HL45168 and HL02212).

REGULATION OF MEMBRANE SKELETON FLUCTUATIONS BY β -ADRENERGIC RECEPTORS

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The observation of fast submicron fluctuations of the cell membrane in erythrocytes and different types of nucleated cells suggests that this phenomenon is a general property of the living cell. These cell surface fluctuations consist of local ($0.25\mu\text{m}^2$), reversible membrane displacements in the range of 0.3-20Hz which reflect the submicron fluctuations of the cortical skeleton. The observation of these fluctuations was detected and monitored by our recently developed Point Dark Field Microscopy.

The present study demonstrates that membrane skeleton dynamics are regulated via membrane receptor transduction pathways. This is illustrated by the modulation of membrane skeleton fluctuations of erythrocytes via the effects of β -adrenergic agonists.

Erythrocytes were preincubated (15min) at 37°C with adrenaline (10^{-5} - 10^{-11}M) followed by measurement of cell membrane fluctuations (CMF's) at room temperature. The incubation with adrenaline at 10^{-9}M caused a 17% elevation of CMF's amplitude (from $14.3 \pm 1.7\%$ ($n=37$) to 16.7 ± 1.9 ($n=33$)). The effect of 10^{-7}M adrenaline caused a saturated and reversible 45% increase of CMF's amplitude (elevation from $14.6 \pm 1.5\%$ ($n=15$) to $21.2 \pm 2.7\%$ ($n=18$), without affecting the shape or size of erythrocytes.

A similar effect was observed in presence of proteranol (10^{-12} - 10^{-7}M), a specific β -adrenergic agonist. Already at 10^{-11}M proteranol a CMF's increase of 25% was observed (from $13.8 \pm 1.2\%$ ($n=15$) to $17.3 \pm 1.7\%$ ($n=21$)).

The transduction of the agonists effects via cAMP was examined by analyzing the direct effect of a membrane permeable derivative of cAMP (8-Br cAMP) on CMF's. 8-Br cAMP (10^{-5}M) caused an elevation of 50% of CMF's amplitude (from 14.1 ± 1.1 (15) to 21.2 ± 2.3 (26)).

CMF's reflect a local bending deformability of a cell which was found to be directly correlated with cell filterability through narrow pores. Thus, the present study suggests the existence of hormonal regulation of microcirculation by modulating membrane skeleton dynamics.

THE ROLE OF PROTEIN 4.1 AND ANKYRIN IN CELL MEMBRANE FLUCTUATIONS OF ERYTHROCYTES

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Fast (0.3-20Hz) submicron local mechanical displacements of the cell membrane, which were observed in erythrocytes and different types of nucleated cells, were shown to regulate cell adhesion kinetics and cell deformability. Cell membrane fluctuations (CMF's) are dependent on the dynamic mechanical characteristics of the membrane skeleton. This time dependent local mechanical fluctuations were measured by our recently developed Point Dark Field Microscopy.

The present study examines the involvement of human erythrocyte membrane-skeleton linker proteins (protein 4.1 and ankyrin) in CMF's.

Protein 4.1, which promotes the actin-spectrin complex and regulates myosin binding to the membrane skeleton, links the skeleton network to the membrane via glycophorin C and band 3. It was shown by Gascard et al., 1993 that inositol phospholipids regulate the binding of protein 4.1 to the glycophorin. The activation of endogenous phosphoinositol phospholipase C (PLC) by calcium ions caused an amplitude decrease of CMF's by 27% (from $14.2 \pm 1.3\%$ ($n=21$) to $10.2 \pm 0.9\%$ ($n=15$)). A similar activation of inositol specific PLC in erythrocyte's open ghosts, which undergo MgATP induced fluctuations, led to a 60% decrease of CMF's amplitude (from $15.0 \pm 1.4\%$ ($n=15$) to $6.3 \pm 1.0\%$ ($n=17$)). Similar results were obtained following the addition of exogenous PLC δ to MgATP fluctuating ghosts. Therefore, it seems that the dynamic association of glycophorin C with protein 4.1 is responsible for local mechanical fluctuations of the cell membrane of human erythrocytes.

Selective dissociation of ankyrin from band 3 protein, carried out by pH elevation from 7.4 to 8.7 (Low et al., 1991), led to a 40% elevated amplitude of erythrocyte's CMF's. Thus, it may be suggested that the the association of ankyrin to band 3 protein constrains CMF's.

EPIDERMAL GROWTH FACTOR INDUCES SERINE PHOSPHORYLATION OF ACTIN

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The cytoskeleton, and especially the actin microfilament system, plays an important role in growth factor induced signal transduction. For example, EGF-receptor kinase activation leads to the phosphorylation of various cytoskeleton associated proteins. Key enzymes in the EGF signal transduction cascade, such as phosphatidylinositol kinase, phosphatidylinositol-4-phosphate kinase, diacylglycerol kinase and phospholipase C are associated with the cytoskeleton. In addition it has been demonstrated that stimulation of cells with EGF or other ligands affects actin organisation. Stimulation of cells by EGF also induces a rapid polymerisation of actin in the cortical skeleton. Co-immunoprecipitation studies and the use of purified components has demonstrated that the EGF-receptor can bind directly to actin via its cytoplasmic domain with no other proteins involved. Other growth factor receptors such as NGF-receptor and PDGF-receptor are also associated with the cytoskeleton.

A number of actin binding proteins, like fodrin, spectrin, tubulin and ezrin have been demonstrated to become phosphorylated upon activation of cells by growth factors. As mentioned above one of the results of stimulation of the EGF-receptor is phosphorylation of various cytoskeletal components. Actin, being a cytoskeletal protein and directly bound to the EGF-receptor is a good candidate for EGF directed phosphorylation. Here we show that actin is phosphorylated *in vitro* and *in vivo* upon EGF stimulation. The phosphorylation occurs on serine and not tyrosine residues and therefore actin appears not to be a substrate of the EGF-receptor itself, but from another kinase whose activity is regulated by EGF through the EGF-receptor.

***Rho* P21 REGULATES THE MOTILITY OF LYMPHOID CELLS
BOTULINUM C3 EXOENZYME INHIBITS LPA-INDUCED
INVASION OF T-LYMPHOMA CELLS THROUGH FIBROBLAST
MONOLAYERS.**

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We use an in vitro model system to investigate the tissue-infiltrating behavior of lymphoid cells. For a panel of cell lines derived from the murine BW5147 T-cell lymphoma, we had previously shown correlations between (1) experimental metastasis formation in mice, (2) in vitro invasion through a fibroblasts monolayer, (3) formation of pseudopodia, and (4) a high level of actin polymerization (J Leukoc Biol 55:552-556; 1994). The role of actin polymerization in the invasive behavior of these lymphoma cells was corroborated by a study with *botulinum* C2 toxin, an ADP-ribosyltransferase that covalently modifies actin and inhibits its polymerization (Eur J Cell Biol, April 1995).

The small GTP-binding proteins *rho* p21 and *rac* p21 are involved in the regulation of the actin cytoskeleton in several cell types. Therefore we have now analyzed the biological effects in our model system of *botulinum* C3 exoenzyme, which specifically ADP-ribosylates and inactivates mammalian *rho* proteins.

BW-O-Li1 is a metastatic, invasive and motile variant of BW5147. In BW-O-Li1 cells treated with C3, *rho* p21 was ADP-ribosylated indeed, and as a result the number of cells invading through a fibroblast monolayer was decreased by more than 60%. Computer assisted analysis of cellular shape changes showed that C3 also inhibited motility of these cells. However, flow-cytometric analysis of FITC-phalloidin stained cells did not reveal any effect on the amount of F-actin.

In serum-starved fibroblasts, LPA (lysophosphatidic acid) is known to induce stress fibre formation via *rho* p21. We found that in the absence of serum, the addition of LPA to BW-O-Li1 cells resulted in a twofold increase of monolayer invasion. Inactivation of *rho* p21 by C3 exoenzyme abolished this stimulation of invasion by LPA. The F-actin content of the cells however was not affected by the treatments with LPA and/or C3.

Our results indicate that the invasive capacity of lymphoid cells can be stimulated via a *rho* p21 mediated signal transduction pathway, without an overall increase in actin polymerization.

MICROTUBULE-ASSOCIATED PROTEINS (MAPs) AND THE TRANSIENT EXPRESSION OF A MAP2c ISOFORM IN DEVELOPING OLIGODENDROCYTES

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The morphological differentiation of oligodendrocytes is characterized by the formation of multiple, microtubule-rich processes which endow these cells with the ability to myelinate many axons simultaneously. Since microtubule-associated proteins (MAPs) strongly influence the structure and function of microtubules, we have investigated their expression in cultured differentiating oligodendrocytes in order to gain insights into MAP function during process formation and stabilization. MAP1B has been compared with two other structural MAPs: MAP4, which is an ubiquitously expressed protein, and MAP2, which hitherto was thought to be confined to neurons and reactive astrocytes. Immunofluorescence microscopy showed that the colocalization of MAP4 with microtubules in oligodendrocyte processes is not as extensive as found previously for MAP1B. Nevertheless, like MAP1B, the expression of MAP4 increases during oligodendrocyte differentiation. In contrast, the expression of MAP2 is transiently elevated in preoligodendrocytes but declines precipitously at the onset of terminal differentiation. Cells of the oligodendrocyte lineage exclusively express a novel isoform of MAP2c which is primarily localized in the cell bodies of preoligodendrocytes. This suggests that MAP2c assists in the initiation of process extension rather than in the stabilization of microtubules in the cytoplasm-filled membranous extensions of mature cells. MAP-tau was not expressed at any developmental stage by oligodendrocytes. The distinct subcellular localizations and patterns of developmental expression of MAP1B, MAP4 and MAP2c suggest that these MAPs have different roles in the regulation of the microtubule network during the differentiation of myelin-forming oligodendrocytes.

DOES THE *DROSOPHILA* ACTIN 5C GENE ENCODE THE

fs(1)polehole GENE? C. R. Wagner¹, C. Myers², H. Salz², A. P. Mahowald³ and K. G. Miller¹, 1) Department of Biology, Box 1229, Washington University, St. Louis, MO. 63130, (314) 935-7339, 2) Case Western Reserve University, Cleveland, OH. 44106, 3) University of Chicago, Chicago, IL. 60637.

The *fs(1)polehole* gene product is one of the components deposited in the oocyte during oogenesis in *Drosophila melanogaster* that is essential for the production of mature, viable eggs. Females homozygous for mutant alleles of *fs(1)polehole* produce allele-specific phenotypes that affect the structure of the eggshell or disrupt embryonic pattern formation. *fs(1)polehole* gene product is required upstream of both the torso receptor tyrosine kinase and the D-raf kinase, which are required for differentiation of the termini of the embryo. *fs(1)polehole* gene product is postulated to have a role in tethering, stabilizing or releasing the torso ligand from its localized source.

Genetically, *fs(1)polehole* is located in the 5C region of the X chromosome. Using a *fs(1)polehole* allele induced by a P-element we have cloned genomic DNA adjacent to the insert. The region encodes four transcripts, *Act5C* and three previously unknown genes. Both genetic and molecular data suggest that one of these transcripts is *fs(1)polehole*. We have shown that transposons expressing three of the transcripts, including the transcript into which the P-element was inserted, do not rescue the female sterility of the *fs(1)polehole* mutants. A transposon that contains the *Act5C* gene was initially able to rescue the *fs(1)polehole* phenotypes; subsequently, we have not been able to repeat the rescue result. Recent experiments indicate that the *Act5C* gene is no longer expressed from the transgene, explaining our inability to repeat the rescue. We are currently generating new transgenic lines that will be tested for both *Act5C* expression and rescue of the *fs(1)polehole* phenotypes. We will present our analysis of this region, including genetic and molecular data that support the idea *Act5C* is *fs(1)polehole*. If *Act 5C* encodes the *fs(1)polehole* gene, we hypothesize that *Act 5C* acts to maintain the integrity of the egg and has a specific role in specifying the termini of the embryo. Such a finding would support the growing body of evidence that the cytoskeleton plays a crucial role in embryonic patterning.

SELECTIN CYTOPLASMIC DOMAINS: THE ROLE OF CYTOSKELETAL INTERACTIONS IN L-SELECTIN ADHESION

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L-selectin is a transmembrane protein that is expressed exclusively on leukocytes. L-selectin functions in binding of leukocytes to lymph node high endothelial venules (HEV) and in leukocyte rolling on the endothelium at sites of infection. Previously it was shown that cells transfected with a version of L-selectin lacking the C-terminal 11 amino acids did not bind to HEV or roll along the endothelium. The purpose of this study was to examine potential cytoskeletal interactions with the cytoplasmic domain of L-selectin and to determine how these interactions might affect adhesion. We measured direct binding of α -actinin, but not vinculin or talin, to the full length cytoplasmic domain of L-selectin using solid phase binding assays. Purified α -actinin bound to L-selectin with a dissociation constant (K_D) of 550 nM. Three findings suggest that there is a complex of proteins that link the cytoplasmic domain of L-selectin to the actin cytoskeleton. The first is that in these solid phase binding assays, talin potentiated binding of α -actinin to L-selectin, although it did not itself bind directly to L-selectin. Second, vinculin bound indirectly to the L-selectin cytoplasmic domain when α -actinin was present. Third, L-selectin co-precipitated with both α -actinin and vinculin, providing in vivo evidence that a complex of cytoskeletal attachment proteins links the L-selectin cytoplasmic domain with the actin cytoskeleton. The truncated L-selectin failed to co-precipitate with α -actinin or vinculin, leading us to conclude that the attachment of L-selectin to the cytoskeleton occurs through the C-terminal 11 amino acids. Immunoelectron microscopic analysis of the wild-type L-selectin and the truncation mutant demonstrated that the mutant localized normally to the cellular microvillar projections. Therefore, the C-terminal 11 amino acids are necessary for the interaction between L-selectin and the cytoskeleton but are not required for proper localization to microvillar projections. We conclude that correct L-selectin receptor positioning alone is insufficient for leukocyte adhesion mediated by L-selectin. Furthermore, it is possible that cytoskeletal interactions with L-selectin play a major role in the inflammatory process that begins with L-selectin-mediated rolling of neutrophils along endothelium.

EFFECT OF OVEREXPRESSION OF NONMUSCLE CALDESMON ON ACTIN FILAMENT DYNAMICS

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The *in vivo* evidence for the function of caldesmon (CaD) in regulating actin filament dynamics in nonmuscle cells is scarce. The mechanism underlying this regulation is not completely understood. We have previously shown that overexpression of an actin-, tropomyosin (TM)-, and Ca^{++} /calmodulin-binding fragment of human fibroblast CaD (CaD39, aa# 244-583) in CHO cells appears to render the actin bundles more resistant to cytochalasin treatment. In addition, analysis of these stable clones with Western blots reveals that stabilized endogenous TM parallels increased amounts of CaD39 expression (Warren et al., J.Cell Biol. 125:359-368, 1994). To further investigate whether this stabilization effect can be also seen in clones expressing full-length CaD (CaD40/39), we have transfected CHO cells with pCB6 vector carrying the full-length cDNA for CaD and isolated several stable clones. Western blot analysis revealed that clones CaD40/39C28, CaD40/39C32, and CaD40/39C6 expressed 1.1, 2, and 4 times more CaD40/39 than endogenous CaD, respectively, whereas CaD40/39C4 expressed only trace amount of CaD40/39. The steady-state levels of endogenous TM4 and TM5 detected by LC24 and CG3 antibodies, respectively, were found to be no change in these CaD40/39 clones. Thus, overexpression of full-length CaD40/39 does not seem to stabilize the endogenous TM, even though the expressed CaD40/39 are found to locate on microfilament bundles. Two possibilities may be used to explain this apparent difference in the ability to stabilize endogenous TM by CaD39 or CaD40/39 overexpression. First, the amounts of CaD40/39 expression in the CaD40/39 stable clones are not as high as that in the CaD39-expressing clones. Thus, no detectable stabilization on endogenous TM can be seen in the CaD40/39 clones. Second, the N-terminal fragment (CaD40, aa# 1-243) may play important role in regulating CaD *in vivo* function. For example, the N-terminal CaD40 may regulate the on-and-off rates of CaD binding to TM-containing microfilaments. The CaD39 fragment would lose this regulation but retain strong binding to TM and microfilaments. As a result, the CaD39 can enhance TM's binding to microfilaments *in vivo* and thus reduce TM's turnover rate and stabilize microfilament bundles.

Characterization of brain-type isoforms of tropomyosin demonstrate weak interactions with filamentous actin *in vitro* and *in vivo*.

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Tropomyosins (Tm) are a group of proteins originally identified in skeletal muscle as a co-regulator of actin-myosin contraction. It is now known that at least 16 different isoforms of Tms are generated from 4 genes in a variety of cell types including neuronal cells. However, little is known about the function of these isoforms in non-muscle cells. In the present study, we have focused on the role of two isoforms expressed in rat brain, TmBr-1 and 3, which are produced via tissue-specific splicing from α Tm gene. Both of these isoforms contain unique C-terminal coding sequences, due to the use of exon 9c. Chick brain also contains the homologous exon, suggesting a conserved function for Tms containing these sequences.

To begin to study the role of these isoforms, we examined if these Tms bind to F-actin. First, we carried out actin-sedimentation assay using recombinant Tms. In this assay, TmBr-1 and TmBr-3 both bound to F-actin, but the affinity was lower compared with other isoforms. Similar results were obtained using either bacteria- and baculovirus-produced Tms, suggesting that the weaker affinity for F-actin is not due to the lack of post-translational modification, such as N-acetylation. Next, we transfected epitope-tagged and untagged TmBr-1 and 3 into fibroblast to examine if these isoforms are incorporated into stress fibers. Consistent with the *in vitro* results, TmBr-1 and 3 were poorly incorporated into stress fibers compared to other isoforms. Co-transfection of other isoforms did not affect this weak binding. Collectively these studies demonstrate that TmBr-1 and 3 have intrinsically weak affinity for F-actin compared to all other non-muscle isoforms tested.

We are also examining the localization of TmBr-1 and/or 3 in neurons using PC12 and rat and chick primary culture.

INTRANEURONAL SORTING OF ACTIN AND TROPOMYOSIN ISOFORMS IS A DEVELOPMENTALLY REGULATED DYNAMIC PROCESS

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Differentiation and maturation of neurons involves a series of profound changes in morphology which are intrinsic to the ultimate function of a neuron. Morphogenesis is most often associated with alterations in the actin based microfilament system of non-muscle cells. We have evaluated whether changes in the composition of microfilaments accompany neuronal morphogenesis. Antisera were raised against the tropomyosin (Tm) isoforms Tm-5 and TmBr-1/-3. In combination with actin isoform specific antibodies, we find that during early morphogenesis *in vivo*, immature growing axons contain β - and γ -actin and Tm-5. In particular, β -actin is enriched in, and Tm-5 exclusively located in, the growing axonal processes relative to the neuronal cell body. β -actin and Tm-5 are lost from mature quiescent axons suggesting that these isoforms are associated with membrane remodelling. The localization of Tm-5 protein to growing axons appears to be achieved by the segregation of its mRNA almost exclusively to the axon hillock and at least the proximal portion of the axon. Cortical neuronal cultures show that the Tm-5 protein is highly concentrated at the base of one process well before a clearly defined axon is observed. The localization of mRNA and protein is not observed for other Tms and suggests a specific role of Tm-5 in the early decisions associated with neuronal polarity.

The loss of β -actin and Tm-5 from axons *in vivo* is accompanied by a progressive appearance of TmBr-1/-3 in axons. The loss of β -actin and Tm-5 from axons involves a redistribution of these molecules to other intracellular sites. Tm-5 becomes associated with the cell body and dendrites whereas β -actin is most prominently localized in synapse-rich structures. At all times, γ -actin is uniformly distributed over all cellular structures. We conclude that both Tm isoforms and β -actin are subject to specific patterns of segregation associated with axonal growth and synaptic maturation. This provides a potential molecular basis for the temporal and spatial specificity of microfilament function during neuronal differentiation. Based on our data we propose a model which suggests associations of specific Tms with specific actin isoforms and a "subunit exchange" scheme of axonal microfilament components during neuronal development that incorporates mRNA localization.

A HUMAN BRAIN TISSUE CULTURE MODEL: ITS APPLICATION IN STUDIES OF THE NEURONAL AND GLIAL CYTOSKELETON.

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Tau and other microtubule associated proteins, bind to and maintain microtubule stability and orientation in both axons and dendrites of central nervous system neurons. However phosphorylated forms of tau are unable to bind microtubules, and form complexes called paired helical filaments. These filaments are the primary components of the neurofibrillary tangles that characterise Alzheimer's disease (1). Recently, the isoforms of apolipoprotein E have been shown to be risk factors for late-onset Alzheimer's disease (2) and are proposed to have differential effects on the rate of tau phosphorylation (3).

To examine the interactions of apolipoprotein E with neurons, specifically human neurons, we have developed a human brain tissue culture model that enables the observation of neuron and glial cell cytoskeletal components by transmission electron microscope and fluorescence microscope.

Cultures of human foetal brain tissue were fixed, permeabilised, immunostained with antibodies against the cytoskeletal components glial fibrillary acidic protein in astrocytes and β -tubulin in neurons, and visualised by fluorescence microscopy. To confirm that the cells had normal ultrastructure, cultures were also prepared and observed by transmission electron microscopy.

Both techniques enable identification and visualisation of the cytoskeletal components of cultured human brain cells. Our pilot studies have shown that cultured human neurons can internalise apolipoprotein E from human sera, and currently we are tracking apolipoprotein E within the cells.

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AN ALPHA-TUBULIN MUTATION SUPPRESSES A. *NIDULANS* NUCLEAR MIGRATION MUTATIONS

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Microtubules and the microtubule motor cytoplasmic dynein are required for nuclei to move along the hyphae of filamentous fungi. Nuclear migration in *Aspergillus nidulans* is blocked by heat-sensitive (hs^-) mutations in the *nudA* gene, which encodes dynein heavy chain, and the *nudF* gene, which encodes a G protein β -subunit-like protein. Hs^- mutations in *nudC* and *nudG* also prevent nuclear migration. To identify additional participants in the process, we isolated extragenic suppressors of a *nudF* mutation. One *nudF* suppressor also suppresses hs^- mutations in *nudA*, *nudC*, and *nudG* and deletion mutations in *nudA* and *nudF*. This suppressor mutation is in *tubA* alpha-tubulin and its characteristics suggest that it destabilizes microtubules. The mutation alters microtubule staining and confers sensitivity to cold and benomyl, two microtubule-destabilizing conditions. Treatment with low concentrations of benomyl also suppresses the hs^- *nudA*, *nudC*, *nudF*, and *nudG* mutations and the *nudA* and *nudF* deletion mutations. Suppression of the hs^- *nudA* and *nudA* deletion mutants is especially interesting because these mutants lack active dynein heavy chain. Together, the results suggest that microtubule destabilization allows nuclei to migrate even in the absence of cytoplasmic dynein motor function.

UTROPHIN N-TERMINUS: ACTIN BINDING AND REGULATION BY CALMODULIN.

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Utrophin is the autosomal homologue of dystrophin with which it shares considerable sequence and presumably functional homology. As an autosomal gene, utrophin is ideally placed to replace defective dystrophin in sufferers of Becker's and Duchenne's muscular dystrophies. We have, therefore, examined the bacterially expressed NH₂-terminal actin binding domain of utrophin for its ability to bind to actin. The NH₂-terminal 261 residues of utrophin (UTR261) bound to skeletal muscle F-actin with an affinity of $19 \pm 2.8 \mu\text{M}$ at a stoichiometry of 2 moles of UTR261 per mole of actin. UTR261 did not bind to monomeric actin, affect actin polymerisation kinetics, and did not cap, sever or crosslink actin filaments. Furthermore, UTR261 bound to platelet actin with a 4-fold higher affinity, $5 \pm 1 \mu\text{M}$. When microinjected into chick embryo fibroblasts, UTR261 was localised to the actin containing stress fibres and focal contacts. From the point of view of actin binding it could be proposed that utrophin could replace dystrophin in dystrophic muscle.

The predicted structure of utrophin (and dystrophin) is related to α -actinin. By this analogy it could be predicted that the C-terminus of utrophin may be able to regulate the NH₂-terminus in a calcium-dependant manner. As a first step toward testing this hypothesis, we have shown that calmodulin can regulate the binding of UTR261 to actin in a Ca^{2+} -dependant manner. The equivalent regions of dystrophin and α -actinin were not regulated by Ca^{2+} /calmodulin. These data suggest that the utrophin-actin interaction is regulatable by Ca^{2+} /calmodulin and (if the COOH-terminus of utrophin binds Ca^{2+}) may be regulated by it's own COOH-terminus by an intermolecular mechanism. Furthermore, these data suggest a fundamental difference between the organisation and regulation of utrophin-actin binding compared to dystrophin. This premise may preclude the therapeutic use of utrophin in muscular dystrophy.

MICROTUBULES ARE INVOLVED IN CYCLIN DEGRADATION AND THE EXIT FROM THE METAPHASE II ARREST IN MOUSE OOCYTES
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To study the role of the metaphase spindle in the exit from the metaphase II arrest, during the period of oocyte activation, mouse oocytes were fertilised or activated parthenogenetically in the presence or absence of the microtubule inhibitor nocodazole. In both cases, nocodazole caused the disappearance of the spindle and prevented the passage of the oocytes into interphase. However, the calcium spiking responses of the oocytes were not affected by nocodazole, being repetitive after fertilisation and a single spike after activation. If, after their activation or fertilisation in nocodazole, oocytes were later removed from the drug, only those that had been fertilised progressed into interphase. This progress was associated with continuing calcium spiking. Moreover, both the spiking and the progress to interphase could be blocked by removal of external calcium or addition of BAPTA-AM. Oocytes that had been activated by ethanol in the presence of nocodazole and then removed from it, to allow reformation of the spindle, only progressed into interphase if given a second exposure to ethanol, thereby eliciting a second calcium transient. Finally, in *Xenopus* oocytes, calcium is involved in cyclin degradation and exit from metaphase through the calmodulin-dependent protein kinase II (CaM K(II)). In mouse oocytes, CaM K(II) is activated by ethanol and nocodazole does not block this activation. These results show that exit from meiotic M-phase requires the simultaneous presence of a fully intact spindle during the release of calcium and that those factors leading to the degradation of cyclin B are only activated transiently. Since cyclin B is being degraded continuously in the metaphase II-arrested mouse oocyte and since this degradation is microtubule-dependent, these data suggest that the superimposition of a high concentration of intracellular calcium is required to tilt the equilibrium further in favour of cyclin degradation if exit from M-phase is to occur.

IDENTIFICATION OF *ARABIDOPSIS* GENES ENCODING
CYTOSKELETAL PROTEINS BY INDUCTION OF CELL SHAPE
CHANGES IN FISSION YEAST

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We have used fission yeast *S. pombe*, as a model system (Edwards et al., PNAS 91,4589,1994), to identify putative cytoskeletal genes of *Arabidopsis thaliana* that may be involved in cell shape maintenance and regulation. An *Arabidopsis thaliana* cDNA library was constructed in pREP3X vector under the inducible nmt1 promoter and transformed into *S. pombe*. When expression of *Arabidopsis* sequences was induced, severe morphological changes were observed in approximately 0.4% of the transformed clones. Thirty clones with various defects in cell shape were further investigated. Sequence analysis of the cDNAs in these clones revealed that several of them appear to encode putative cytoskeletal proteins including homologs of myosin heavy chain, myosin-like protein, α -2 and α -6 tubulin genes, and verprolin etc. Overexpression of the other genes also caused changes in cell shape and these genes include homologs of ubiquitin-conjugating enzyme, Ran GTPase activating protein, RAD26, *A. thaliana* MYB2 etc., as well as a few novel genes. Characterization of these cDNA sequences in terms of their expression patterns and roles in plant cell morphogenesis is in progress.

SIGNAL TRANSDUCTION IN WILD TYPE AND MYOSIN MUTANT
CELLS DURING MULTICELLULAR MORPHOGENESIS OF
Dictyostelium

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During starvation, *Dictyostelium* amoebae generate pulses of cAMP which are relayed outward from an aggregation center. Cells respond by moving toward the center in a pulsatile manner, forming streams and then aggregates. The pulsatile movement of cells can be visualized in time lapse as an expanding wave when aggregation is observed using phase contrast microscopy. Previous analysis has shown that the interval between waves is about 7 minutes. Using fluorescent labeling, confocal microscopy and computer-assisted motion analysis, we are able to monitor individual wild-type and myosin null mutant (*mhcA*⁻) cell behavior during development and correlate this information with the rate of wave propagation obtained from phase contrast images. Our results show:

1. Wild-type cells initially move with a periodicity of 9 minutes, at a mean rate of 4 $\mu\text{m}/\text{min}$ and a peak velocity is 10 $\mu\text{m}/\text{min}$. As streams form, these cells move with a periodicity of 7 minutes, a mean rate of 5.2 $\mu\text{m}/\text{min}$ and a peak velocity is 13 $\mu\text{m}/\text{min}$. At later stages cells move at 3.5 minutes intervals, at an average of 8 $\mu\text{m}/\text{min}$, and a peak velocity of 17 $\mu\text{m}/\text{min}$.
2. Myosin mutant (*mhcA*⁻) cells show periodic movement at 11 minutes interval and a mean rate of 1.0 $\mu\text{m}/\text{min}$ early in development and a peak velocity is 3.5 $\mu\text{m}/\text{min}$. In most cases, *mhcA*⁻ cells do not form streams. Sometime, they may form very fragile streams which then break up into small aggregates. The interval of movement for cells in such streams prior to break-up is 7 minutes, and the cells move at 1.7 $\mu\text{m}/\text{min}$ with a peak velocity is 5 $\mu\text{m}/\text{min}$.
3. When 2% labeled wild-type cells are mixed with *mhcA*⁻ cells, wild-type cells move faster than the mutants and accumulate rapidly at the aggregation center. The mean speed for labeled wild-type cells is 3.3 $\mu\text{m}/\text{min}$ and the peak velocity is 10 $\mu\text{m}/\text{min}$. The pulse interval measured from phase waves is 7 minutes for these mixed aggregates.

Our data reveal that the interval of cAMP pulsing increases as development proceeds for both wild-type and myosin mutant cells. The slower pulsing of mutants is an unexpected consequence of the myosin mutation and may be related to the slower movement of the mutant cells. Our preliminary data suggests that in a mixing experiment, wild-type cells which accumulate at the aggregation center may gradually increase the pulse rate of mixed aggregates.

IDENTIFICATION OF α -SYNTROPHIN BINDING TO
SYNTROPHIN TRIPLET, DYSTROPHIN, AND UTROPHIN

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Syntrophin represents three cytoplasmic components of the dystrophin-glycoprotein complex which links the cytoskeleton to the extracellular matrix in skeletal muscle. α -Syntrophin has now been translated *in vitro* and shown to directly associate with all three components of the syntrophin triplet and with dystrophin. The *in vitro* translated 71-kDa non-muscle dystrophin isoform, containing the cysteine-rich/C-terminal domain, can also interact with not only the syntrophin triplet but also a α -syntrophin fusion protein containing C-terminal 328 amino acids. The syntrophin binding motif in dystrophin was localized to exons 73 and 74 including amino acids 3447-3481 by comparing the interactions of ³⁵S-labeled α -syntrophin and seven overlapping human dystrophin fusion proteins. Binding data suggested that more than one syntrophin interaction site in this binding motif. α -Syntrophin also directly interacts with a C-terminal utrophin fusion protein. α -Syntrophin is localized to the muscle sarcolemma as well as to the neuromuscular junction in control mouse muscle. However, similar to utrophin, α -syntrophin is only present at the neuromuscular junction in *mdx* mouse muscle in which dystrophin is absent. Our data suggest that α -syntrophin binds all syntrophin isoforms, and syntrophin directly interacts with dystrophin through more than one binding site in dystrophin exons 73 and 74 including amino acids 3447-3481.

MODULATION OF SHIGELLA LOCOMOTION IN PtK-2 HOST CELLS BY A LISTERIA ACTA OLIGO-PROLINE PEPTIDE.

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Intracellular locomotion of *Listeria monocytogenes* requires the surface protein ActA, and microinjection of sub-micromolar concentrations of the second oligo-proline repeat CFEFPPPPTDE of ActA completely arrests *Listeria* rocket-tail assembly and intracellular bacterial motility [Southwick and Purich (1994) *Proc. Natl. Acad. Sci.* 91, 5168-5172]. As observed with time-lapse, phase-contrast video microscopy, *Listeria* and *Shigella flexneri* traverse the cytoplasm of PtK2 host cells with similar rates and trajectories. While *Shigella* motility in host cells requires the surface protein IcsA, there is no sequence homology between ActA and IcsA. Because *Shigella* IcsA protein is devoid of oligo-proline stretches, we addressed the question: Does *Shigella* recruit a host cell protein containing oligoproline repeats to function in place of the ActA protein? In support of this possibility, we found that *Shigella* migration is inhibited by the oligo-proline ActA analogue over the same intracellular concentration range (80-800 nM) that blocked *Listeria*. [speed before injection was $0.06 \pm .03$ $\mu\text{m}/\text{sec}$ (SD, n=36); speed after injecting 80 nM ActA peptide was $0.00 \pm .01$ $\mu\text{m}/\text{sec}$ (SD, n=37). Because profilin is the only actin cytoskeletal protein known to interact with poly-L-proline, we examined the effects of injecting equimolar binary solutions of the peptide analogue and profilin. Significantly, injection of the binary solution: (a) neutralized the analogue's inhibitory action; (b) stimulated previously non-motile bacteria to commence locomotion; and (c) increased the observed rates of intracellular motility by a factor of four. The mean speed was 0.05 ± 0.02 $\mu\text{m}/\text{sec}$ (n=32) before injection and 0.2 ± 0.1 $\mu\text{m}/\text{sec}$ (n=60) after introducing 80 nM profilin plus 80 nM ActA analogue. Microinjection of profilin alone had minimal effect [e.g., mean speed of 0.13 ± 0.02 $\mu\text{m}/\text{sec}$ (n=12) before injection and 0.14 ± 0.07 $\mu\text{m}/\text{sec}$ (n=14) after injection of 80 nM profilin]. These findings suggest profilin in PtK2 cells may be present in several pools and that motility is promoted by a submicromolar pool tethered at oligoproline-binding sites. Our findings also suggest *Shigella* attracts host cell oligo-proline-containing protein(s) to its surface to mimic the action of the *Listeria* ActA protein and thereby provide the driving force for intracellular *Shigella* movement. Such host cell protein(s) may also participate in the dynamic regulation of actin assembly at the leading edge of nonmuscle cells. (Sponsored by NIH Research Grant AI34276)

ASSEMBLY AND DYNAMICS OF HUMAN CENTROMERES.

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Centromeres act in mitosis to specify the association of microtubule-dependent motor proteins with the chromosome and to mechanically link the bipolar spindle halves into a functional mitotic apparatus. CENP-A is a human autoantigen that represents a centromere-specific homologue of the core nucleosomal protein histone H3. Like histone H3, CENP-A contains two domains, a unique N-terminal tail and a C-terminal histone fold domain that shares 75% similarity with histone H3. To understand the basis for the specific assembly of CENP-A at centromeres, a series of CENP-A:histone H3 chimeras have been prepared and assayed for their ability to assemble at centromeres in HeLa cells. Previous experiments showed that the histone fold domain is required for targeting to centromeres. We have now examined the role of individual secondary structural elements within CENP-A, aided by the 3.1 Å structure of histone H3 within the histone octamer. The data demonstrate that two segments in the central portion of the histone fold domain are required for assembly at centromeres, a long central helix present in the core of the octamer and a surface loop-beta strand (strand A) motif that lies across the DNA binding path of the nucleosome. In contrast, a second loop-strand motif (strand B), which contacts DNA at the nucleosome dyad axis, is not required for targeting CENP-A. Due to the symmetry of the nucleosome, there are two strand A elements on the surface of the histone octamer, suggesting a model in which cooperative interactions at two sites on the DNA are important for the specific assembly of CENP-A at centromeres.

A second human autoantigen, CENP-B, has been used to create a novel bioluminescent probe of centromere structure and dynamics *in vivo*. CENP-B is a sequence-specific DNA binding protein that recognizes the alphoid DNA component of human centromeres. A chimeric gene was prepared by linking the DNA binding domain of CENP-B to the green fluorescent protein of *A. victoria* (GFP), to create a fluorescent centromere DNA binding protein, CB-GFP. Cells transfected or microinjected with this construct express a fluorescent product that is localized at centromeres in HeLa cells, demonstrated by immunofluorescence using anti-centromere antibodies. Time-lapse fluorescence microscopy using a cooled CCD camera demonstrates that cells labeled with CB-GFP remain competent to undergo mitosis, allowing direct visualization of centromere movements in mitotic cells. In addition, preliminary data using fixed cells indicate that the morphology of CB-GFP labeled centromeres changes during mitosis. Centromeres appear to become elongated during prophase and metaphase, as if they are being stretched by the bipolar forces exerted by the spindle. CB-GFP thus appears to be reporting not only the spatial distribution and motility of centromeres, but also on the forces exerted across the centromere during chromosome congression and metaphase.

CENP-E KINETOCHORE-ASSOCIATED KINESIN IS REQUIRED FOR CHROMOSOME ALIGNMENT AND BALANCE OF KINETOCHORE FORCES. Tim J. Yen¹ and B.T. Schaar², ¹ Fox Chase Cancer Center Philadelphia, Pennsylvania 19111; ² Cell Biology Graduate Group, University of Pennsylvania, Philadelphia, Pennsylvania 19104.

CENP-E is a 312 kDa human kinetochore protein that is a member of the kinesin superfamily of microtubule-based motor proteins. Its presence at kinetochores during mitosis suggests that it may be a motor that is responsible for one or more aspects of chromosome movement. We have directly tested the function of CENP-E at kinetochores by depleting it from kinetochores. Our approach was to microinject affinity-purified CENP-E antibodies into the cytoplasm of interphase HeLa or U2-OS cell lines. Since CENP-E is a soluble cytoplasmic protein during interphase, the injected antibodies should form complexes that might interfere with subsequent assembly onto the kinetochore after nuclear envelope breakdown. Injected cells progressed into mitosis on schedule but were arrested in mitosis (>8 hours). Inspection of the mitotically blocked cells showed that bipolar spindle formation was unaffected, nor the localization of several known centromere-kinetochore proteins. However, chromosomes were found collapsed around either one of the poles and scattered between the two poles. Examination of the behaviour of the unaligned chromosomes by DIC microscopy showed that the mono-oriented chromosomes never exhibited any significant motion or migrated away from the pole. The chromosomes located between the poles establish bipolar attachments as they exhibit oscillations. These oscillations are highly irregular and the chromosomes never achieve a tight metaphase alignment but meander throughout the spindle. In cells blocked for many hours, all of the bipolar attached chromosomes are separated along the length of their arms and are only held together by connections at the telomeres. This separation is not a result of precocious anaphase since the mono-oriented chromosomes in the same cell are not separated. Furthermore, some cells overcome the mitotic block and proceed into anaphase whereby the telomeric connections of these unzipped chromosomes synchronously dissolve and the chromatids migrate towards opposite poles.

We propose that CENP-E functions as a kinetochore motor to propel mono-oriented chromosomes away from its pole so that it can establish a bipolar attachment with the opposite pole. Once a bipolar attachment is made, CENP-E must contribute to the overall balance of kinetochore-generated force. When CENP-E is depleted from kinetochores, a poleward force predominates which may be responsible for ripping apart the chromatid arms.

GENETIC APPROACHES TO DETERMINING THE INTERPHASE AND MITOTIC FUNCTIONS OF THE FISSION YEAST SPINDLE POLE BODY.

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As with most eukaryotic cells, the *S.pombe* microtubular cytoskeleton undergoes two alterations during progression through the mitotic cell cycle, once at the beginning and again at the end of mitosis. During interphase microtubules extend through the cytoplasm from one end of the cell to the other. They are nucleated independently of the nuclear-membrane-bound mitotic MTOC, the spindle pole body (SPB). Upon mitotic commitment interphase microtubules depolymerise and new ones polymerise from the nuclear faces of the duplicated SPBs and a typical spindle forms as the microtubules extending from either SPB interdigitate. After separation of the daughter nuclei during anaphase B the SPBs are inactivated and an interphase array is re-established.

We have identified three genes, which, when mutated, can block spindle formation. One of these, *cut7⁺*, is a member of the microtubule motor kinesin heavy chain gene superfamily, whilst the products of *sad1⁺* and *cut12⁺* appear to be involved in SPB function.

The *sad1⁺* (spindle architecture defective) gene encodes a protein with a predicted Mol Wt. of 58 kD, which migrates as multiple bands in SDS PAGE. The amino terminal third of the protein is highly acidic and is followed by a 19 amino acid hydrophobic stretch. These are followed by a p34^{cdc2} phosphorylation consensus site immediately adjacent to a sequence that exhibits weak homology to an EF Hand motif. The migration of Sad1 protein on SDS PAGE is affected by calcium levels in the gel. *sad1⁺* is essential and immunofluorescence microscopy shows that Sad1 protein localises to the SPB during all stages of the fission yeast life cycle. When overproduced in the mitotic cycle the protein localises to the nuclear periphery in addition to the SPB.

Cut12 is also implicated in SPB function as one of the major phenotypes in *cut12.1* mutants is that the levels of Sad1 at each SPB varies and the more brightly staining SPB is often unable to nucleate microtubules. Further evidence for a link between Cut12 and Sad1 functions is suggested by the ability of multicopy plasmids containing the *sad1⁺* gene to suppress the *cut12.1* defect. The DNA sequence of the *cut12⁺* gene predicts a 58.5 kD protein with two coiled coil domains.

Parallel studies using anti-Sad1 antibodies as a marker for the SPB have demonstrated a role for the SPB in determining the position of the interphase nucleus.

Kar1p IS A COMPONENT OF THE HALF BRIDGE OF THE SPINDLE POLE BODY OF *S. cerevisiae* AND INTERACTS WITH Cdc31p

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The spindle pole body (SPB) is the microtubule organizing center (MTOC) of *S. cerevisiae*. Despite their importance during the cell cycle, especially in mitosis, relatively little is known about the composition of MTOCs. Several components, like γ -tubulin and centrins, are conserved in a large variety of organisms, indicating their important role in the function of the MTOC. The centrin of *S. cerevisiae* is Cdc31p, an essential, Ca^{2+} -binding protein, which has been localized to a substructure of the SPB, the so-called half bridge (Spang et al., 1993. J. Cell Biol., 123:405-416). Kar1p was thought to be involved in SPB duplication (Rose and Fink, 1987. Cell, 48:1047-1060). We (Spang et al., 1995. J. Cell Biol. in press) and Biggins and Rose (1994. J. Cell Biol., 125:843-852) identified Kar1p as a Cdc31p interacting protein. Using an *in vitro* assay, we could demonstrate, that Cdc31p interacts specifically with a short sequence in the central part of Kar1p. This sequence shows similarities to calmodulin binding motifs. However, there are three negatively charged amino acids which are not found in calmodulin binding peptides. An overproduced Kar1- β -galactosidase fusion protein has been localized to the outer plaque of the SPB (Vallen et al., 1992b. Cell, 68:505-515). This raises the question, how Cdc31p and Kar1p may interact when they are located to different substructures of the SPB. Therefore, we decided to determine the subcellular localization and the cell-cycle distribution of wild-type Kar1p by immunoelectron microscopy and immunofluorescence. Kar1p was detected at the half bridge of the SPB. Using synchronized cells, we looked at the cell-cycle dependent localization and observed that Kar1p is associated with the SPB during the entire cell cycle, which is also in contrast to the behavior of the overproduced Kar1- β -galactosidase fusion protein. Further studies revealed that Cdc31p is absent from the half bridge in *kar1* cells, while Kar1p is still associated with the SPB in *cdc31* cells.

SPINDLE POLE BODY DYNAMICS IN LIVING YEAST CELLS.

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In the budding yeast *Saccharomyces cerevisiae*, the spindle pole body (SPB) serves as the MTOC and is the functional analog of the centrosome of higher organisms. By expressing a fusion of a yeast SPB-associated protein, Nuf2p, to the *Aequorea victoria* green fluorescent protein (GFP), we were able to observe the SPBs by fluorescence microscopy in living cells undergoing mitosis. Using a cooled CCD imaging system, we made movies of dividing cells during the 15 - 30 min period of anaphase from the localization of the short spindle at the bud neck through the breakdown of the elongated spindle. Our observations indicate that anaphase in yeast proceeds in four steps defined by the movements of the spindle and the rate of spindle elongation. These four sequential activities of the spindle are: 1) Alignment at the mother-daughter junction, 2) "fast" elongation, 3) spindle translocation in the absence of elongation, and 4) "slow" elongation.

Prior to elongating, the short spindle changes its orientation in a series of apparently random movements until it is properly aligned at the bud neck. This activity of the spindle had not been described previously, and we refer to it as alignment. Within 30 seconds of finding the "proper" orientation, the SPBs separate at a constant (10 sec measurement intervals), average rate of $1.48 \mu\text{m}/\text{min}$ ($n = 10$; $SD = 0.11$) over a period of 100 - 150 seconds until the spindle has grown to 50-60% the length of the mother. During this period, the spindle is located mostly in the mother. We refer to this period as fast elongation. Following fast elongation, spindle elongation stops, and the entire spindle appears to translocate as a unit. This activity appears to contribute to the movement of the daughter's SPB into the daughter cell. We refer to this period as spindle translocation in the absence of elongation, since some spindle translocation also occurs during the period of fast elongation. Following translocation of the spindle into the bud, a second phase of spindle elongation is initiated but at a slower rate, ($0.69 \mu\text{m}/\text{min}$; $n = 7$; $SD = 0.14$) than the earlier period of fast elongation.

PLACEMENT OF THE DIVISION SITE IN FISSION YEAST

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Cell division requires the proper spatial and temporal regulation of the assembly of the contractile actin ring. Early studies in cytokinesis have suggested a model in which the contractile ring is positioned by an inducing signal emanating from the spindle asters. We have taken a molecular genetic approach in the fission yeast *S. pombe* to analyze these processes. The contractile actin ring in fission yeast may be positioned by an analogous signal emanating from the premitotic nucleus or spindle pole body. A large genetic screen has identified six genes required for actin ring assembly, and one required for actin ring placement. The product of one of these genes, the *cdc12*⁺ gene product, is a candidate for a component of the inducing signal. *cdc12*⁺ is essential for actin contractile ring formation and cell division, but is not required for organization of actin cortical patches during interphase. Localization of *cdc12* using a GFP-*cdc12* fusion demonstrates that *cdc12* may be present at the spindle pole body in the middle of the cell during interphase and translocates to the contractile ring at the cell surface during mitosis. Thus, *cdc12* may link the position of the contractile ring with the position of the interphase spindle pole body. *cdc12* is a large proline-rich protein that binds SH3 domains and shares sequence similarity with the *S. cerevisiae* bud site selection protein Bni1, the *Drosophila* cytokinesis protein diaphanous and the vertebrate formins. The *mid1*⁺ gene product is required for positioning the actin ring in the middle of the cell and thus may be involved in the accurate transmission of the inducing signal.

OVEREXPRESSION OF THE C-TERMINUS OF THE INTERMEDIATE
CHAIN OF CYTOPLASMIC DYNEIN CAUSES A MITOTIC BLOCK IN
DICTYOSTELIUM

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Cytoplasmic dynein is thought to play a role in the retrograde transport of organelles as well as in the formation and orientation of the mitotic spindle. The cytoplasmic dynein heavy chains (HC) contain the microtubule binding and ATPase sites. The role of the dynein associated subunits is still unclear, although it has been suggested that they may function in the binding of dynein to its cargo. We previously cloned and characterized the 80 kDa dynein intermediate chain (IC) of *Dictyostelium discoideum* (1993, Mol. Biol. Cell 4:47a). The *Dictyostelium* IC is most similar to the rat 74 kDa IC and the axonemal oda6 IC in the C-terminus of the molecule, suggesting that this domain may be responsible for a conserved function such as binding to dynein HC. We tested this hypothesis by expressing the conserved and divergent domains of the IC in wild type *Dictyostelium* cells.

Myc-tagged, full-length IC (ICmyc), the divergent N-terminal domain (IC-Nmyc), and the highly conserved C-terminal domain (IC-Cmyc) were expressed in AX3 cells under control of an inducible promoter. Immunoprecipitation experiments indicate that full-length ICmyc successfully competes with endogenous IC for binding to the HC and does not change the ATPase activity of isolated ICmyc dynein. IC-Nmyc does not associate with the HC, but forms part of a 9S complex that includes endogenous IC not bound to HC, suggesting that the N-terminus of the IC is involved in subunit-subunit interactions. IC-Cmyc is able to associate with the dynein HC at a 1:1 molar ratio with the endogenous IC, but is lethal at higher levels of expression. These data suggest that the IC contains at least two functional domains. DAPI staining and tubulin immunofluorescence analysis indicate that maximally induced IC-Cmyc cells have a 6 to 7-fold higher mitotic index than uninduced IC-Cmyc cells with 70% of mitotic cells blocked in prophase. We speculate that overexpression of IC-Cmyc is lethal to cells because it binds the HC, but lacks the proposed "cargo-binding" domain, rendering dynein functionally inactive. These results indicate that IC function is required for an essential role of cytoplasmic dynein in the formation of the mitotic spindle in *Dictyostelium discoideum*. Supported by NIH Grant GM39264 to RLC.

Purification and Characterization of a γ Tubulin Complex from *Xenopus laevis*

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γ tubulin is a wide spread and highly conserved protein first discovered in *Aspergillus nidulans*. It is found to be a component of microtubule organizing centers (MTOCs) in all organisms examined so far. Genetic and cell biological studies suggest that γ tubulin functions as a microtubule nucleator at the MTOC. We found that the majority of γ tubulin in the *Drosophila melanogaster* early embryo exists as two discrete complexes with similar S values of about 20S but different stokes radii. In the unfertilized *Xenopus* eggs, however, the majority of γ tubulin is found to be in one complex. We have purified the single *Xenopus* γ tubulin complex in its native form. It contains six major proteins besides γ tubulin. One of these proteins is α tubulin. γ tubulin is the only protein that binds to radioactively labeled GTP in the protein complex. The γ tubulin complex forms a ring structure of ~28.5nm in diameter and it promotes microtubule assembly in vitro.

CENTROSOME ASSEMBLY: THE ROLE OF PERICENTRIN.

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Pericentrin is a highly conserved coiled-coil protein of centrosomes that seems to play an important role in the organization of the microtubule cytoskeleton (Doxsey et al., Cell, 76, 639, 1994). Results from recent functional experiments both in vitro and in vivo suggest that pericentrin serves as a template for the assembly of microtubule nucleating material. When a coiled coil segment lacking both N- and C- terminal globular domains is overexpressed cytoplasmic foci are formed. These foci contain the overexpressed (HA-tagged) protein, endogenous pericentrin, γ -tubulin, MPM-2 antigens but not α -tubulin or actin. This phenotype is not observed when the N- and C-terminal globular domains are overexpressed. We conclude that the truncated coiled domain, which dimerizes in vitro, assembles into foci and specifically recruits centrosome components from the cytoplasm. Ectopic assembly of these centrosome-like structures from the truncated pericentrin molecule may result from loss of regulatory information, such as phosphorylation sites known to control assembly of other coiled coil proteins. Several such sites are lacking in the truncated molecule.

In another set of experiments, pericentrin antibodies were covalently coupled to fluorescently-labeled beads and added to cytoplasmic extracts from *Xenopus* eggs together with rhodamine-labeled tubulin. After a five minute incubation, microtubules were seen emanating from the antibody-beads. The five minute lag period is consistent with the time required for centrosome assembly in other systems (Doxsey et al., Cell, 76, 639, 1994, Stearns and Kirschner, Cell, 76, 623, 1994). Spontaneous microtubule assembly was not observed and microtubules were not detected when other antibodies were used. These data suggest that pericentrin is concentrated at the bead surface and recruits other centrosome components or that it is concentrated at the bead in a preformed complex with other centrosome proteins.

LOCALIZATION OF ACTIN RELATED PROTEIN ARP-1 TO CELL DIVISION REMNANTS IN EARLY *C. ELEGANS* EMBRYOS

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The *C.elegans* actin related protein Arp-1 is an apparent homolog of the *S.cerevisiae* ACT5 and the vertebrate Act-RPV. ACT5 is essential for alignment of the mitotic spindle along the mother-bud axis, and is thought to function in the same pathway as yeast dynein (Muhua Li *et al*, Cell 78 (1994) 669. Act-RPV is a component of the dynactin complex which stimulates dynein to transport vesicles along microtubules (Lees-Miller *et al*, Nature 359 (1992) 244.

Anti-ACT5 antibodies cross react with *C. elegans* Arp-1 as well as conventional actins on immunoblots. Indirect immunofluorescence of fixed *C. elegans* embryos with these antibodies shows discrete dot shaped staining located at all mid-bodies/Cell Division Remnants (CDRs); no cross-reactivity to either cytoplasmic or muscle actin is detected. At the first cell division, Arp1 is detected towards the completion of cytokinesis, such that it is located at the CDR between the daughter cells, AB and P1. The staining persists through the P1 prophase, during which the P1 centrosome-pair undergoes a 90° rotation. Laser microsurgery experiments have shown that a site near the location of CDR is necessary for P1 centrosome rotation (Hyman, J.Cell Biol., 109(1989)1185. The proximity of the laser sensitive site to the location of Arp-1 suggests that Arp-1 might be involved in rotation, perhaps as a part of a dynactin complex. Additionally, two other components of the dynactin complex, actin, and capping protein, transiently accumulate at the CDR (Waddle *et al*, Development 120(1994)2317) during P1 centrosome rotation. In addition to staining the AB/P1 CDR, anti-Arp1 antibodies stain all CDRs in the AB and P1 lineages in early embryos. Some of the CDRs do not abut centrosome rotations, suggesting that Arp-1 might have additional or alternative functions common to both AB and P1 lineages.

Centrosome rotation in P1 occurs during prophase. At increasing frequencies after completion of P1 prophase, the dot shaped Arp-1 staining disappears from the cell surface and appears in the cytoplasm of P1, close to the anterior centrosome of P1. Two markers for CDR: anti-kinesin antibodies and phalloidin, also colocalize with this internal Arp-1 staining. These results suggest that the entire CDR might come to reside in the interior of P1. Centrosome rotations also occur in the blastomeres EMS and P2. Once again, Arp-1 staining appears in the interior of EMS and P2 after rotations are complete. Examination of live *C.elegans* embryos has revealed movements of unusual vesicular structure(s) from the surface of P1 and EMS into their respective interiors. The appearance of Arp-1 staining initially on the surface and later in the interior seems to follow the same temporal and spatial pattern as the vesicular movement suggesting that Arp-1, and perhaps the CDR, is associated with one of these vesicular structures.

AMOEBOID MOTILITY WITHOUT ACTIN: INSIGHTS INTO THE MECHANISM OF LOCOMOTION DERIVED FROM THE *ASCARIS* MAJOR SPERM PROTEIN (MSP)

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The amoeboid sperm of nematodes such as *Ascaris suum* do not require either actin or myosin to crawl over solid substrates. Instead, in these cells, the role usually assigned to actin has been taken by the major sperm protein (MSP), which assembles into filaments that pack the sperm pseudopod. These MSP filaments are organized into multi-filament arrays called fiber complexes that assemble along the the leading edge of the pseudopod and flow rearward in a pattern that is intimately associated with motility. Under appropriate conditions purified MSP can be induced to form either crystals or filaments. Examination of this material by EM and image processing revealed that the basic unit of the MSP cytoskeleton is a two-stranded helical filament. Each subfilament within a filament is itself constructed from a helically-wound strand of MSP monomers. Filaments associate further to form higher-order helical arrays, called macrofibers, thus indicating that direct interaction between complete filaments is an intrinsic property of MSP. Crystals of MSP appear to be constructed from helical arrays of molecules that have the same symmetry observed in subfilaments and, thus, are suitable for studying both the structure of the molecule and the interaction geometry involved in self-assembly.

Mixture of sperm cytosol with a detergent extract of cell membranes in the presence of ATP also induces MSP polymerization but in this case the filaments bundle into multifilament arrays, readily visible by light microscopy, that have the same organization and dynamic properties as the fiber complexes that comprise the sperm cytoskeleton. These arrays, like fiber complexes, grow only at one end and frequently branch as they elongate. The growing end of each array labels intensely with anti-phosphotyrosine antibody as does the leading edge of the sperm pseudopod where fiber complexes are assembled. Thus, protein tyrosine phosphorylation may be involved in regulating the vectorial assembly of the MSP motile apparatus observed both in vivo and in vitro. Together, structural characterization of the MSP system and reconstitution of key elements of the motile apparatus in vitro have given important insights into the fundamental molecular mechanism of amoeboid motility and have emphasized the importance of vectorial filament assembly and of filament bundling to locomotion. Supported by NIH GM29994.

ACTIN FILAMENT POPULATIONS IN THE FISH KERATOCYTE

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We recently showed that actin filaments in the keratocyte cytoskeletons produced by Triton extraction display an ordered orthogonal arrangement subtending an angle of around 45° to the cell front, and extend up to several microns through the lamellipodium. Now we have investigated the F-actin content of keratocytes using quantitative video microscopy of phalloidin-labeled cells after various fixations. Analysis of the intensity distribution of fluorescent phalloidin staining across the lamellipodium revealed a steep gradient of staining from the front to the rear of the lamellipodium for cells first fixed and then extracted with Triton. Extraction followed by fixation produced a flatter phalloidin profile, in which staining was preferentially reduced by approximately 45% in the anterior part of the lamellipodium. We have also used phase contrast video microscopy, and fluorescent video microscopy in conjunction with microinjection of fluorescent actin and found evidence in support of radial circumferential flow at the rounded lateral edges of these cells and retraction of actin bundles at the rear of the cell, consistent with the findings of Lee et. al. (Nature 362 167-171, 1993). Evidence of tension across the rear of the cell came from the observation that disruption of the cytoskeleton to either side of the nucleus by micromanipulation leads to its instantaneous retraction in the opposite direction.

We postulate that there are two populations of actin filaments in the lamellipodium; a Triton extractable population comprised of shorter filaments on the dorsal surface toward the front of the lamellipodium, and a less extractable, on average longer subset of ventrally located filaments reaching further back into the lamellipodium. It is proposed that the rhodamine phalloidin intensity gradient reflects the gradient of filament lengths.

These and other data are further interpreted within the context of a treadmilling type mechanism of actin dynamics which delivers filaments via circumferential flow to the rear of the cell, where they participate in a myosin-based contractile system necessary for the maintenance of cell shape and the generation of nuclear traction. A model of cell movement incorporating these different features will be presented.

CELLS LACKING OR OVEREXPRESSING THE DICTYOSTELIUM 30,000 DALTON ACTIN BUNDLING PROTEIN EXHIBIT ABNORMALITIES IN CELL STRUCTURE AND DEVELOPMENT.

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The Dictyostelium discoideum 30,000 dalton actin bundling protein is a calcium regulated actin cross-linking protein recruited to the filopodia, phagocytic cup, cleavage furrow, and sites of cell to cell contact. The function of the protein has been investigated using homologous recombination to create cells lacking the protein, and overexpression to create cells with greater than normal quantities.

The gene replacement vector was assembled by insertion of the hygromycin resistance cassette into a 4 kB genomic clone encoding the Dictyostelium 30,000 dalton actin bundling protein. Following transformation and selection for growth in the presence of hygromycin, cells lacking the 30,000 dalton protein were identified using Western blotting. The recombination event was further verified by Northern blotting and PCR analyses on genomic DNA. The cells can grow in suspension in axenic media, and on plates with E. coli B/r as food. These cells spread to cover a larger area of substrate, and make more and longer filopodia stained with rhodamine-phalloidin than do the parental AX-2 cells. Moreover, the cells lose bits of cytoplasm which break off during locomotion on the substrate, so that the substrate becomes littered with cellular debris. Finally, in chemotaxis experiments, cells lacking the 30,000 dalton protein can orient toward a source of cAMP in a micropipette, but move more slowly than wild type AX-2 cells.

Cells overexpressing the 30,000 dalton protein were prepared by inserting sequences encoding the protein into the vector pVEII, so that expression driven by the discoidin promoter begins coincident with the initiation of development. Developing cells have ~5 fold more of the 30,000 dalton protein than do wild type AX-3 parental cells. The development of the overexpressing cells is aberrant in that development is significantly delayed, and both the slugs and fruiting bodies are abnormal.

The results indicate that the 30,000 dalton protein is not essential for growth, but does make profound contributions to cell structure, motility, and morphogenesis.

MUTANTS LACKING MYOSIN II CANNOT RESIST FORCES GENERATED DURING MULTICELLULAR MORPHOGENESIS

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We have used fluorescent labeling, confocal microscopy and computer assisted motion analysis to observe and quantify individual wild-type and myosin II mutant cell behavior during early multicellular development in *Dictyostelium discoideum*. Wild-type cells move at average rates of $8.5 \pm 4.9 \mu\text{m}/\text{min}$ within aggregation streams and can exhibit regular periodic movement at 3.5 minute intervals. Myosin II mutants (*mhcA*⁻) under the same conditions move at $5.0 \pm 4.8 \mu\text{m}/\text{min}$ and failed to display regular periodic movement. When removed from aggregation streams *mhcA*⁻ cells moved at only $2.5 \pm 2.0 \mu\text{m}/\text{min}$, while wild-type cells under these conditions moved at $5.9 \pm 4.5 \mu\text{m}/\text{min}$.

Analysis of cell morphology reveals that *mhcA*⁻ cells are restricted to the edges of aggregation streams and are grossly and dynamically deformed within wild-type aggregation streams but not when removed from streams and examined in isolation. The high velocity of the mutant cells results from their being passively dragged by wild-type cells. In double labeling experiments, wild-type cells can be observed to periodically attach to mutant cells, drag and distort them and then detach and move away. The distortion of the mutants is likely caused by a flaccid cortex that is unable to resist the tension exerted by adhered wild-type cells. The segregation of the mutant cells to the edges of the streams is likely to be due to the inability of mutant cells to generate sufficient protrusive force to penetrate the tightly adhered wild-type amoebae. When myosin mutants are mixed with cells lacking the adhesion protein gp24, the streams are less tightly adhered, the myosin mutant cells are more normal in shape and they are no longer segregated to the edges of the streams. Elimination of the adhesion protein gp80 has no effect on this process.

The consequences of the loss of myosin II for cells during multicellular development are much more severe than has been previously revealed for isolated cells. Myosin appears to play an important role in maintaining cortical integrity when cells are subjected to external compression and distention forces. The data are consistent with the hypothesis that a major function of myosin is the generation and maintenance of cell shape.

ACTIN FILAMENT ASSEMBLY IS INDUCED BY THE
BACTERIAL PATHOGEN *LISTERIA MONOCYTOGENES* IN
FRACTIONATED EXTRACTS FROM *XENOPUS LAEVIS*
EGGS AND HUMAN PLATELETS

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Diverse species of pathogenic bacteria including *Listeria monocytogenes*, *Shigella flexneri* and *Rickettsia rickettsii* move intracellularly and spread from cell to cell by inducing the assembly of a tail composed of cross-linked and bundled actin filaments. The force for propulsion is thought to be provided by directed polymerization of actin filaments at the bacterial cell surface. Assembly of an actin tail by *Listeria* requires the bacterially synthesized cell surface protein ActA and multiple host proteins, including actin and profilin. However, pure actin and profilin together are not sufficient to support bacterial induced actin assembly, indicating that additional host factors are needed.

It was previously shown that *Listeria* motility can be reconstituted in unclarified cytoplasmic extracts from *Xenopus laevis* eggs (Theriot et al. 1994, Cell 76: 505), permitting biochemical dissection of the mechanism by which bacteria promote actin assembly. To create a more tractable system for the isolation of host factors needed for actin assembly we have reconstituted *Listeria* motility in high speed supernatants from *Xenopus* egg extracts and human platelet extracts. In all extracts tested, *Listeria* can assemble tails in the presence of the nonionic detergent triton X-100, suggesting that membrane associated factors are not essential.

We have fractionated high speed supernatants from human platelet and *Xenopus* egg extracts by column chromatography and have assayed for fractions that allow bacteria to induce actin assembly. In both extract systems an S-sepharose binding fraction is sufficient, in the presence of exogenously added actin, to support tail assembly. Further fractionation may identify additional host proteins necessary for different aspects of tail formation.

REGULATION OF THE MECHANOCHEMISTRY AND ENZYMOLOGY OF CALMODULIN-BINDING MYOSINS

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Myosins constitute a rapidly expanding superfamily of mechanoenzymes that can currently be divided into at least 10 distinct classes based on sequence analysis of the force-producing head domain in the N-terminus of the heavy chain. Joined to the myosin head is a neck domain that contains the sites for binding light chains. This region is continuous with a unique C-terminal tail domain that may aid in cargo attachment or localization of a particular myosin to membranes. Myosins have also been broadly classified as being either conventional or unconventional. This latter group constitutes the vast majority of identified myosins and is often distinguished by the ability to bind multiple calmodulin light chains.

A common theme observed for calmodulin-binding myosins is that their mechanocatalytic and enzymatic activities are dramatically altered by micromolar calcium *in vitro*. In the case of chick brain myosin-V and brush border (BB) myosin-I, calcium results in the activation of MgATPase activity concomitant with either a decrease or complete cessation of mechanocatalytic potential. This response is completely dependent on Ca^{2+} -binding to the calmodulin light chains. Moreover, this marked increase in MgATPase is observed for myosin stripped of one or more light chains by elevated Ca^{2+} , or following calmodulin removal by proteolytic severing of the neck region from native myosin.

Calmodulin-binding myosins may also be regulated by methods similar to conventional myosin-II. For example, BB myosin-I MgATPase and *in vitro* motility are inhibited by muscle and nonmuscle tropomyosin isoforms. In contrast, chick brain myosin-V motility is unaffected by skeletal tropomyosin.

We are currently using antibodies to immunoprecipitate novel myosins for mechanocatalytic characterization using a *Limulus* acrosomal process and laser tweezer-based *in vitro* motility assay. This assay should be useful for mechanocatalytic analysis of scarce myosins.

THE WHEN AND HOW OF MOLECULAR MOTOR BINDING TO GOLGI MEMBRANES.

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We have previously shown that Golgi membranes isolated from intestinal epithelial cells possess the actin-based motor myosin-I, the MT minus-end directed motor cytoplasmic dynein and its *in vitro* motility activator p150/Glued (J.C.B. 126:661 (1994)). Because more than 50% of dynein is soluble and Golgi stacks lack dynein, whereas TGN38/41-containing small membranes possess dynein, it is likely that dynein binding to membranes must be regulated. In an attempt to study the regulation of dynein binding to membranes we have used an *in vitro* Golgi budding assay. In the presence of cytosol and ATP, isolated stacks bud small dynein-containing vesicles that are enriched in sialated proteins and the apically targetted protein alkaline phosphatase. Dynein therefore appears to bind to areas of stacks destined to bud: dynein is present on budded membranes, but is absent from the stacks after budding. To determine if dynein was associated with coated or uncoated Golgi vesicles, budding assays were performed in the presence of GTP γ S, which has been shown to cause an accumulation of COP-coated vesicles, or NEM, which has been shown to cause the accumulation of uncoated vesicles. Membranes budded in the presence of NEM contained 10-fold more dynein than controls, while GTP γ S treatment had no effect on dynein binding. Our results suggest that dynein binds to Golgi membranes during late stages in protein-membrane transport through the Golgi. We have also begun studying the mode of association of these motors with Golgi membranes. In contrast to the large amount of soluble dynein in these cells, essentially all myosin-I is associated with actin or membranes. Thus far, no membrane receptor for either dynein or myosin-I has been well documented. All GPI anchored proteins are directly apically targetted in these cells and are resistant to cold Triton X-100 extraction. In the Golgi small membrane preparation, ~50% of the dynein and all of the myosin-I is Triton insoluble as is ~70% of the GPI-anchored protein alkaline phosphatase. Interestingly, dynein binds to Triton extracted or peripheral membrane protein-stripped Golgi stacks. These results suggest that dynein and myosin-I associate with rafts of glycosphingolipid-enriched membrane domains containing a potential receptor. Our working model in polarized epithelia is that dynein transports vesicles from the Golgi and the basolateral membranes along MTs to the cell cortex where myosin-I provides local delivery through the actin-rich cytoskeleton to the apical membrane.

DISRUPTION OF THE DYNEIN-DYNACTIN INTERACTION INHIBITS AXONAL TRANSPORT IN EXTRUDED SQUID AXOPLASM

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Vertebrate dynactin is a 20S complex of ten different polypeptides, which was first identified by copurification with the microtubule motor protein, cytoplasmic dynein. *In vitro* assays have suggested that dynactin is required for cytoplasmic dynein-driven, microtubule-based motility of vesicles, and recent genetic and biochemical studies suggest that dynactin and cytoplasmic dynein are capable of a direct interaction. However, there is no evidence that the dynein-dynactin interaction is required for organelle transport *in situ*. To approach this question, we have examined the effect of antibodies to p150^{Glued} on vesicular transport in extruded axoplasm of the giant axon from *L. pealei*. Two rabbit polyclonal antibodies raised against rat p150^{Glued} cross-reacted with a 160 kDa band on western blots of squid axoplasm or optic lobe cytosol. Immunoprecipitation from optic lobe cytosol with these antibodies coprecipitated polypeptides of 94, 55 and 45 kDa, the latter of which reacted with anti-centractin antibodies. In addition, in whole cytosol, both the 160 and 45kDa immunoreactive polypeptides cosedimented on a sucrose gradient solely as a ~20S complex. These data demonstrate that our antibodies react with the squid homologue of p150^{Glued}. When extruded axoplasm was treated with ~1 mg/ml of either of the anti-p150^{Glued} antibodies, vesicle transport, as observed by AVEC-DIC microscopy, was fully inhibited in the bulk axoplasm. In dissociated regions of axoplasm where vesicle movement on individual microtubules could be analyzed, vesicle velocity was decreased to ~0.5 $\mu\text{m}/\text{sec}$ after 30 min of treatment while the untreated control maintained a velocity of ~2.0 $\mu\text{m}/\text{sec}$. Rigor binding of vesicles to individual microtubules was observed. A non-cross reactive antibody had no effect on axonal transport. We have recently demonstrated that a p150^{Glued} affinity column is capable of selectively retaining cytoplasmic dynein from whole rat brain cytosol, via direct interaction between p150^{Glued} and the dynein intermediate chain (Karki et al., in preparation). To examine the nature of the inhibitory effect of our antibodies on vesicular transport in the axon, a p150^{Glued} affinity matrix was preadsorbed with anti-p150^{Glued} antibodies prior to passing squid optic lobe cytosol over the matrix. Control columns (either untreated or preadsorbed with BSA) retained cytoplasmic dynein from the cytosol, while on columns pretreated with either of the anti-p150^{Glued} antibodies, dynein binding was reduced. Taken together, our results suggest that an interaction between cytoplasmic dynein and the dynactin complex is required for axonal transport. (Supported by NIH GM48661).

THE CONTRACTILE VACUOLE COMPLEX OF *Dictyostelium*.

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Dictyostelium amoebae contain a remarkable osmoregulatory organelle, the contractile vacuole complex. We have visualized the organization of this organelle by indirect immunofluorescence using antibodies against proteins enriched on contractile vacuole membranes. Heuser and Morisaki have monitored the dynamics of the system in living cells by interference reflection microscopy. Both approaches indicate that the contractile vacuole system is highly dynamic and pleiomorphic. Under hyperosmotic conditions (or after emptying), the membranes exist as a tubular labyrinth. Under hypoosmotic conditions, large vacuoles are formed, apparently by incorporation of membrane that was previously tubular. The number of vacuoles varies from one or two to several dozen, depending on the cell type and its stage in the cell cycle. Whatever their number, all vacuoles are capable of fusing with the plasma membrane and expelling their contents, arguing that all parts of the system are pluripotent.

The major protein component of contractile vacuole membranes is a vacuolar H^+ -ATPase. Using monoclonal antibodies against two subunits of the *Dictyostelium* V-ATPase, we have cloned the corresponding cDNAs. The predicted amino acid sequence of the *Dictyostelium* 100-kD subunit is 45% identical to a transmembrane subunit of the rat clathrin-coated vesicle/synaptic vesicle V-ATPase; that of the 68-kD subunit is 69% identical to the catalytic A subunit of bovine V-ATPase. We are using molecular genetics to examine the function of these subunits. An attempted knockout of the gene encoding the 100-kD subunit has uncovered the presence of a second closely related gene, now being analyzed. No transformants have been obtained in which the A-subunit gene has been inactivated, suggesting that this subunit is essential. However, we have transformed cells with a vector containing the 5'-end of the A-subunit gene in an antisense orientation, driven by an inducible promoter. Conditions leading to expression of the antisense transcript result in many cells rounding up and detaching from the substratum. Immunofluorescence indicates that the rounded cells are deficient in the 68-kD antigen. Their shape may reflect increased tension in the cortical cytoskeleton, helping to protect the cells from osmotic damage.

Another protein greatly enriched on contractile vacuole membranes is calmodulin. We have identified and partially characterized a 150-kD calmodulin-binding protein associated with vacuolar membranes. This protein cross-reacts with anti-peptide antibodies against *Acanthamoeba* myosin IC. However, a 2-kb cDNA clone that appears to encode the amino terminal half of the *Dictyostelium* 150-kD protein shows no significant homology to myosin or any other known protein. We are currently seeking to clone the carboxy terminal half of this gene, and to explore the function of the 150-kD protein through molecular genetics.

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NUCLEAR MEMBRANE DYNAMICS AND SORTING DURING MITOSIS

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Despite recent advances, the mechanism of nuclear membrane disassembly and reassembly during mitosis remains elusive. A factor contributing to current uncertainties is that the disassembly products and the vesicular precursors of the nuclear envelope have not been sufficiently characterized.

We have previously shown that mitotic vesicles enriched in ER membrane proteins and enriched in nuclear lamin B and lamin B "receptors" dock on vimentin intermediate filaments (IFs). Using high resolution confocal microscopy, we have now mapped the location of these vesicles relative to IFs and chromatin at the various phases of mitosis using synchronized CHO cells. Consistent with the idea that the lamin B-carrying vesicles represent genuine precursors of the nuclear envelope, our observations show that the mitotic vesicles largely colocalize with vimentin bundles in prometaphase, gradually detach from the IFs until anaphase and assemble around chromatin in late telophase.

Exploiting these observations, we have mass-isolated lamin B and lamin B-"receptor" containing vesicles from synchronized mitotic cells using magnetic beads coupled to anti-vimentin antibodies. Employing in vitro reconstitution assays and morphometric analysis, we could show that native mitotic membranes prepared in this manner have the capacity to capture chromosomes and fuse into large cisternae along the surface of chromatin fragments. Chromosome capturing does not occur in the presence of octadecanoic acid, is cytosol-dependent and is promoted by exogenously added phosphatases. Interestingly, stripping the membranes from peripheral membrane proteins abolishes their chromosome binding capacity, whereas reconstitution with nuclear lamin B restores binding to chromosomes but not homotypic fusion around chromatin.

Guided by these findings, we propose a unifying mechanism for the reversible disassembly of the nuclear membranes during cell division. The central points of this mechanism are that vesiculation of the inner and outer nuclear membrane occurs independently at the onset of mitosis and that, after binding transiently to IFs, inner nuclear membrane vesicles are targeted to chromosomes in a lamin B-dependent way.

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MOLECULAR ANALYSIS OF *DROSOPHILA* NUCLEAR LAMIN HEAD-TO-TAIL POLYMERIZATION

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To gain more detailed insight into the role of polymerization in nuclear lamin function we analyzed head-to-tail polymerization of *Drosophila* lamin Dm₀ at the molecular level.

A co-immunoprecipitation assay was developed to study binding between *Drosophila* lamin Dm₀ fragments. Association between NH₂- and COOH-terminal fragments exhibited the same dependence on pH and ionic strength as determined previously by electron microscopic analysis of head-to-tail polymerization of full length chicken lamin B₂. Observation of interacting fragments by electron microscopy showed directly that binding was indeed head-to-tail. Like head-to-tail polymerization, binding was inhibited by phosphorylation with cdc2 kinase. Phosphorylation of the conserved residue serine 50 with cAMP dependent kinase inhibited binding as well.

Using the two-hybrid system we demonstrated that the two lamin fragments also bound each other in vivo in yeast nuclei. Mutant fragments were generated by PCR-mediated random mutagenesis and selected for loss of binding. Most mutations were found in the short NH₂- and COOH-terminal end segments of the rod domain which are conserved among all intermediate filament proteins. These regions were previously shown to be essential for polymerization of cytoplasmic intermediate filament proteins into 10 nm filaments.

Two of the point mutations (R⁶⁴->C and I³⁹⁶->V) were introduced into the full length lamin. As predicted, these mutant lamins failed to form head-to-tail polymers. Our data indicate that a common binding motif, probably involving an overlap between the NH₂- and COOH-terminal ends of the rod domain, is used in polymerization of nuclear lamins and cytoplasmic intermediate filament proteins. Results facilitate analysis of the role of head-to-tail polymerization in lamin function.

**SPECIFIC PEPTIDE INHIBITORS OF CYTOSKELETAL
INTERMEDIATE FILAMENT (IF) ASSEMBLY: EFFECTS ON CELL
SHAPE AND PHYSIOLOGY.**

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Over the past few years it has become apparent that IF are not static cytoskeletal components, but rather they are in a state of dynamic equilibrium. This latter state results from the rapid exchange between IF subunits and polymers. *In vitro* it has been demonstrated that synthetic peptides, whose sequences are derived from the most highly conserved domains of IF proteins can efficiently disassemble IF at 1:1-1:3 (IF: peptide) molar ratios (see Steinert *et al.* [1993], BBRC, 197:840-848). This disruption of IF polymers is most likely due to the competitive binding of the peptides to sites involved in the protein-protein interactions important in maintaining IF structure. Recently we have also shown that these peptides have no detectable effects on F-actin or microtubule structure at similar molar ratios *in vitro*. These observations have led us to attempt to employ IF peptides as specific disrupters of IF structure and function *in vivo*. For this purpose we have chosen to use peptides whose sequence is derived from the helix initiation 1A subdomain of the central rod portion of human keratin 10 or human vimentin. The *in vivo* experiments involve the microinjection of these 1A peptides into live cells. Within a few minutes after microinjection, dramatic changes begin to take place in IF networks ultimately resulting in their disassembly. Coincident with this disassembly process, both fibroblasts and epithelial cells are altered from a flattened asymmetric shape to a rounded one. During these peptide induced changes, normal organelle distribution is altered and microtubules and microfilament bundles are disassembled. The effects on IF as well as the other two major cytoskeletal systems appear to be completely reversible. The results indicate that 1A peptides are powerful tools for studying IF structure and function *in vivo* and that IF play a central role in cell shape maintenance, as well as in the interactions among the major cytoskeletal systems. (Supported by NIH GM36806).

NEUROFILAMENTS, AXONAL GROWTH AND MOTOR NEURON DISEASE

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Neurofilaments are the most abundant cytoskeletal polymers in myelinated axons, particularly in the largest caliber axons such as those of the α motor neurons. Several lines of evidence, including analysis of transgenic mice expressing altered levels of the three individual neurofilament subunits, have combined to prove that neurofilament accumulation is essential for the radial growth of axons that normally takes place after stable synapses have formed. This is an important event because it is crucial for establishing normal conduction velocity. Mechanistically, growth in caliber requires both NF-L (to support filament assembly) and NF-H, whose heavily phosphorylated tail domains extend from the filament core. Despite this important normal function, an abundance of evidence has demonstrated that aberrant accumulation of neurofilaments may cause human motor neuron disease, including amyotrophic lateral sclerosis (ALS). First, abnormal accumulations of neurofilaments are an early hallmark of the pathogenic process. Second, forced overexpression of neurofilament subunits in transgenic mice leads to motor neuron dysfunction. Third, expression of modest levels of a point mutant in neurofilament subunit NF-L causes massive, selective degeneration of spinal motor neuron cell bodies and axons. In the few surviving motor neurons, perikaryal and axonal accumulations of neurofilaments are prominent. The neuronal pathology is accompanied by severe denervation-induced atrophy of skeletal muscles. Lastly, human ALS caused by dominant mutation in the enzyme superoxide dismutase (SOD1) is characterized by massive neurofilamentous accumulations essentially identical to that produced by neurofilament mutations. These efforts prove that neurofilament mutations can cause selective motor neuron death and strongly support the hypothesis that abnormalities in neurofilaments are key pathologic intermediates in many examples of human motor neuron disease.

INDIRECT ASSOCIATION OF EZRIN WITH β ACTIN: ISOFORM-SPECIFICITY AND CALCIUM-SENSITIVITY.

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Whereas it has been demonstrated that muscle and nonmuscle isoactins are segregated into distinct cytoplasmic domains, the mechanism regulating subcellular sorting is unknown. To reveal whether isoform-specific actin-binding proteins function to coordinate this event, cell extracts derived from motile (E_m) vs. stationary (E_s) cytoplasm were selectively and sequentially fractionated over filamentous isoactin affinity columns prior to elution with a KCl step gradient. A polypeptide of interest, which binds specifically to β actin filament columns, but not to muscle actin columns has been conclusively identified as the ERM family member, ezrin. We studied ezrin- β interactions *in vitro* by passing extracts (E_m) over isoactin affinity matrices in the presence of Ca^{2+} -containing vs. Ca^{2+} -free buffers, with or without cytochalasin D. Ezrin binds and can be released from β actin Sepharose-4B in the presence of Mg^{2+} /EGTA and 100 mM NaCl (at 4°C and room temperature), but not when affinity fractionation of E_m is carried out in the presence of 0.2 mM $CaCl_2$ or 2 μ M cytochalasin D. N-acetyl-(leucyl)₂-norleucinal and E64, two specific inhibitors of the calcium-activated protease, calpain I, protect ezrin binding to β actin in the presence of calcium. To test the hypothesis that ezrin binds directly to β actin, we performed three sets of studies under a wide range of physiological conditions (pH 7.0-8.5) using purified pericyte ezrin and either α or β actin. These included co-sedimentation, isoactin affinity fractionation and co-immunoprecipitation. Results of these experiments reveal that purified ezrin does not directly bind to β actin filaments, either in solution or while isoactins are covalently cross-linked to Sepharose-4B. This is in contrast to our finding that ezrin and β actin could be co-immunoprecipitated or co-sedimented from E_m -derived cell lysates. Biochemical analyses are focused on revealing the putative β actin filament capping activity that facilitates ezrin-isoactin associations.

VINCULIN CONFORMATION REGULATES LIGAND BINDING

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Recently, we have reported the ability of vinculin to interact with F-actin and α -actinin as a function of vinculin conformation. The actin binding site of vinculin, located between residues 893-1016 within the 27kD carboxyterminal tail, confers F-actin binding to vinculin in solution and in cell models (Menkel et al., *J. Cell Biol.* 126, 1231, 1994). By comparing genuine vinculin with recombinant chimaeric proteins comprising only the vinculin tail fragment, we could demonstrate that the tail without the head piece is efficiently recruiting actin filaments in vitro as well as in transfected cells. Thus, the presence of the 90kD head may exert an inhibitory effect on the vinculin-F-actin interaction. Similar observations have recently been described by Johnson and Craig (*Nature* 373, 261, 1995). Complementary to these results, we found that the α -actinin binding site located within the N-terminal head piece can be negatively regulated by the presence of the carboxyterminal tail (Kroemker et al., *FEBS Lett.* 355, 259, 1994). These studies suggest a model where the two large parts of the vinculin molecule, which are linked through a proline-rich hinge, mutually control ligand binding.

To obtain more structural information on vinculin which might challenge this model, we have performed dynamic light scattering studies on the conformation of vinculin under physiological conditions. This analysis expands and completes previous work on intact vinculin and the isolated head (Eimer et al., *J. Mol. Biol.* 229, 146, 1993). Our results provide new data on the dimensions of the head and tail fragments and several parameters of their interactions.

This work supports the view that vinculin may grossly alter its conformation to select for specific ligands, a process that may also be used by other cytoskeletal proteins within the focal adhesion.

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CONDENSATION OF MYOSIN II MINIFILAMENT ASSEMBLIES DURING CELL PROTRUSION.

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Formation of new myosin II structures in protruding cells was followed on light microscopic and ultrastructural levels. In protruding REF 52 fibroblasts microinjected with tetramethylrhodamine-myosin II, we have observed the formation of new myosin spots close to the active edge or directly on the edge of lamellipodium during its partial retraction. Later on, these spots elongated to assume a striking morphology of long (up to 10 μ m) wavy ribbons of uniform width. Eventually ribbons aligned to form a parallel periodic pattern across actin filament bundles similar to arrangement of A-bands of muscle sarcomeres. Before the alignment of ribbons was complete, it was often possible to observe a single ribbon associated with randomly organized actin, or with one or several small bundles of actin filaments, suggesting that myosin organization may precede and induce actin organization. To determine the molecular arrangement of myosin in ribbons, we have performed correlative light and electron microscopy of cytoskeletons depleted of actin to directly visualize myosin filaments as well as cytoskeletons labelled for myosin by immunogold procedure. It was found that myosin ribbons consisted of bipolar minifilaments bound to each other at their head-containing ends and arranged in a single row as a tight parallel stack or a loose zig-zag-like conformation. Sometimes filaments in neighboring ribbons made frequent contacts to each other resulting in a transformation of ribbon-like organization into two-dimensional network of myosin filaments. Degree of myosin filament alignment in ribbons and networks increased in parallel with development of actin filament bundles. Myosin ribbons were particularly pronounced in REF 52 cells, but networks and smaller ribbons were found also in other mammalian cells. We propose that small zig-zag-like aggregates of myosin filaments induce the formation of actin bundles by pulling on actin filament network and that alignment of actin and myosin filaments proceeds via folding of myosin filament aggregates in an accordion-like fashion.

ROLE OF THE ACTIN CYTOSKELETON IN REGULATED MITOCHONDRIAL MOVEMENTS DURING YEAST CELL DIVISION

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The actin cytoskeleton is required for polarized yeast cell growth and cell division. During this process, mitochondria and other organelles are transferred from the mother cell to the developing bud. Time-lapse fluorescence microscopy was used to study the role of the actin cytoskeleton in mitochondrial movement during yeast mitosis. Mitochondria in living yeast were visualized using two methods: membrane potential sensing dyes and Green Fluorescent Protein targeted to mitochondria. Labeled mitochondria are resolved as tubular organelles aligned in radial arrays that converge at the bud neck. Mitochondria in the central region of the mother cell move linearly towards the bud, traverse the bud neck, and progress towards the bud tip at an average velocity of $4.9 \times 10^{-2} \pm 2.1 \times 10^{-2} \mu\text{m}/\text{sec}$. In contrast, mitochondria in the peripheral region of the mother cell and at the bud tip display little to no movement. This pattern of immobilization and polarized mobilization contributes to the efficiency of mitotic mitochondrial inheritance. Yeast strains containing temperature sensitive mutations in the actin-encoding *ACT1* gene show abnormal mitochondrial distribution. No significant mitochondrial movement is evident in these mutants at permissive temperatures and after short-term shift to semi-permissive temperatures. Thus, the actin cytoskeleton is important for normal mitochondrial movement during inheritance. These studies are consistent with previous findings that mitochondria co-localize with actin cables in yeast, and that isolated yeast mitochondria display actin- and ATP-dependent motor activity. Current efforts focus on the effect of myosin mutations on mitochondrial movement and inheritance.

ISOFORM SPECIFIC COMMUNICATION BETWEEN ACTIN AND TROPOMYOSIN MULTIGENE FAMILIES

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Contractile proteins are encoded by multigene families, subsets of which are coexpressed in different cell types. In muscle progenitor cells, myoblasts, non-muscle actin and tropomyosins form the backbone of the cytoskeleton whereas muscle isoforms are produced upon myoblast differentiation into myotubes. Manipulation of actin isoform expression in myoblasts has previously been observed to impact on tropomyosin isoform expression. This has led us to test the ability of precocious myoblast expression of the muscle isoforms of actin to influence expression of other muscle contractile proteins. Transfection of the human cardiac actin gene into C2 myoblasts produces stable cell clones which accumulate substantial levels of both the encoded mRNA and protein. The cells display no change in cell shape or contractile protein gene expression.

Transfection of the human skeletal actin gene produces C2 clones with at most, 5% of the mRNA level of adult skeletal muscle. In contrast to cardiac actin, the skeletal actin transfectants induce high levels of muscle tropomyosin isoform mRNAs and proteins. The mouse cardiac actin gene is also induced and smooth muscle α actin and desmin are both upregulated to the level found in myotubes. Control transfections using skeletal-actin promoter-CAT constructs and deletion of the actin coding region have no impact on C2 cells. This suggests that the skeletal actin protein is capable of inducing the expression of muscle tropomyosins and cardiac actin. We conclude that the temporal order of actin isoform expression is important during myogenesis. Precocious expression of skeletal actin has induced the myoblast to prematurely acquire a partially differentiated phenotype. This suggests the existence of communication pathways which directly link the expression of isoforms between different contractile protein gene families.

RHO, RAC AND CDC42 GTPASES REGULATE THE ASSEMBLY OF PLASMA MEMBRANE FOCAL COMPLEXES ASSOCIATED WITH THREE DISTINCT TYPES OF ACTIN STRUCTURE.

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Rho and rac, two members of the ras-related superfamily of small GTPases, regulate distinct signal transduction pathways linking extracellular growth factors to the polymerisation of actin in cells. Activation of rho leads to the formation of stress fibres while rac activation stimulates actin polymerisation at the plasma membrane producing lamellipodia and membrane ruffles. We have now identified a function for cdc42, another member of the rho family; microinjection of cdc42 into subconfluent Swiss 3T3 fibroblasts leads to the rapid formation of a third type of actin-based structure found at the cell periphery, filopodia. Time lapse video recordings reveal that around 20-30 filopodia are induced on each cell within 5 minutes of injecting cdc42. The filopodia are highly motile often dissociating from and reattaching to the substratum and can grow up to around 10-25µm in length over 30 minutes.

In addition to stress fibers, rho controls the assembly of focal adhesion complexes which are regions of close contact between the cell and the underlying substrate and the sites at which actin stress fibres are attached to the plasma membrane. We have now shown that rac and cdc42 also stimulate the assembly of multi-molecular focal complexes at the plasma membrane. Activation of rac leads to the assembly of focal complexes distributed around the entire leading edge of the lamellipodium whereas microinjection of cdc42 leads to the formation of focal complexes at the cell periphery and along and at the tips of growing filopodia. These complexes are distinct from and formed independently of rho-induced focal adhesions.

Finally, the activities of the three GTPases are linked to each other in a hierarchical fashion such that activation of cdc42 in Swiss 3T3 cells leads to the sequential activation of rac and then rho. This suggests a molecular model for the co-ordinated control of cell motility by members of the rho family of GTPases.

ROLES OF SRC, RAS AND RAC IN THE MORPHOLOGICAL CHANGES OBSERVED IN MIGRATING PC12 CELLS

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Stationary cells are induced to migrate during the course of a wide range of biological processes, yet the morphological changes and signaling pathways that are involved in this switch are not fully understood. Previously, we have reported that concurrent addition of nerve growth factor (NGF) and phorbol 12-myristate,13-acetate (PMA) rapidly elicited migration in PC12 cells grown on laminin (J. Neurosci. Res. 1980, 25:453-462). By using time-lapse video microscopy, we have now identified cell spreading, ruffling with pinocytosis and cell-rear retraction as major components of the morphological change that elicits motile behavior in these cells. Within five minutes of NGF and PMA addition, the cells spread along their entire circumference, producing a circular lamella. After spreading, the cell membrane starts to ruffle at several points along this lamella and produce pinocytotic vesicles that are later recycled to the plasma membrane. Retraction of several cell-substratum attachment points at the periphery of the cell forms its trailing edge. As a result, PC12 cells acquire a lamellipodia, become polarized and start migrating at speeds that are comparable to those of other migrating cells.

We have investigated the roles of src, p21ras and rac, a small GTP-binding protein, in mediating these structural changes in PC12 cells. When src and Protein Kinase C (PKC) are activated concurrently, the cells display the same morphological behavior as they do upon NGF and PMA addition, suggesting that activation of a src-like kinase is responsible for NGF action. Activated ras only partially synergizes with PKC signaling. When ras is inhibited, the number of migrating cells is greatly decreased due to a defect in spreading and retraction. The rac-expressing cells grow lamellipodia in a time- and extracellular matrix-dependent manner, but there is limited synergy between rac and PKC activation. Expression of a dominant negative form of rac inhibits cell spreading and motility in a manner very similar to inhibition by a dominant negative form of ras. From these findings, we conclude that the cytoarchitectural changes induced by NGF and PMA are mediated by src, ras and rac. We suggest that ras and rac activation contribute to motility at more downstream levels of signaling than does src activation.

ACTIVATION OF ADHESION-DEPENDENT SIGNAL TRANSDUCTION VIA CYTOSKELETAL PERTURBATION.
Formation of focal adhesions and actin bundles, tyrosine phosphorylation of FAK and paxillin, and induction of DNA synthesis in serum-starved cells after microtubule disruption.

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Cell adhesion to the extracellular matrix was shown to generate transmembrane signals which induce tyrosine phosphorylation of specific set of proteins. Serum starvation reduces the level of tyrosine phosphorylation of these proteins leading to disruption of actin cables and focal adhesions. Addition of serum or specific growth factors restore normal actin cytoskeleton structure and increases tyrosine phosphorylation of focal adhesion proteins, paxillin and FAK. We have shown that microtubule disruption in serum-starved Swiss 3T3 cells induces events which are similar to those induced by serum stimulation. Both nocodazole and vinblastine, microtubule disrupting agents with different mechanisms of action, promoted rapid formation of numerous actin cables and increase of size and number of vinculin-containing focal adhesions. Simultaneously, tyrosine phosphorylation of focal adhesion proteins, in particular, paxillin and FAK increased significantly. Genistein, an inhibitor of protein tyrosine kinases, decreased the level of tyrosine phosphorylation in affected cells and inhibited the nocodazole-induced tyrosine phosphorylation of paxillin and FAK. This treatment also inhibited formation of focal adhesions and actin cables in nocodazole-treated cells. Another drug, H-7, an inhibitor of protein serine/threonine kinases which blocks cell contractility (Volberg et al., *Cell Motility and Cytoskel.* 29: 321-338), also prevented nocodazole-induced assembly of focal adhesions and actin cables and the increase of tyrosine phosphorylation of paxillin and FAK, but did not affect tyrosine phosphorylation of other proteins. These data suggest possible involvement of tension-related events in the stimulation of actin reorganization and tyrosine phosphorylation. As short-term effects of microtubule disruption are similar to those of serum or mitogenic factors, we examined also the effects of transient microtubule depolymerization on the DNA synthesis in affected cells. In agreement with observations of other authors we have shown that 3 h nocodazole treatment stimulates DNA synthesis. Moreover, we have demonstrated that both genistein and H-7 efficiently prevent this stimulation when added simultaneously with nocodazole. Together these data show that microtubules control tyrosine phosphorylation and (or) actin cytoskeleton in serum-starved cells. Disruption of this control leads to the reorganization of actin and burst of tyrosine phosphorylation that in its turn stimulates DNA synthesis, without addition of external ligands.

THE INTRAMOLECULAR ASSOCIATION OF VINCULIN HEAD AND TAIL DOMAINS : A POTENTIAL MECHANISM FOR REGULATING ADHERENS JUNCTION ASSEMBLY

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Progress in understanding cellular regulation of adherens junctions will require correlation of the action of signalling cascades induced by ligation of cell adhesion receptors with specific effects on the structures or interactions of the proteins which localize to these sites. Toward this goal, we are attempting to define the mechanisms by which vinculin localization at adherens junctions is controlled. Through analysis of the structural basis for gain of ligand binding activities in proteolytic domains of vinculin, we have uncovered a potential mechanism for regulation of vinculin recruitment and function. Utilizing protease protection, chemical cross-linking and direct binding studies, we have found a tight interaction ($K_d \sim 50$ nM) between the amino-terminal, 95-kDa head and the carboxy-terminal, 30-kDa tail domains of vinculin, which occurs intramolecularly in the native molecule. Talin and the tail domain compete for binding to vinculin head domain, demonstrating that loss of the intramolecular interaction of head and tail accounts for increased affinity for talin of the head domain compared with intact vinculin. This intramolecular association also masks an F-actin binding site present in the carboxy-terminal domain. Cosedimentation and low shear viscosity assays, and direct visualization by transmission electron microscopy, reveal an interaction ($K_d \sim 1$ μ M) between F-actin and the vinculin tail domain. Vinculin itself neither cosediments with nor cross-links F-actin. The 95-kDa head fragment of vinculin, but not intact vinculin, inhibits both cosedimentation and cross-linking. Kinetic analysis of F-actin depolymerization reveals that the vinculin tail domain induces a phalloidin-like stabilization of actin filaments which is completely inhibited by the head domain. These data demonstrate that the vinculin tail domain contains an F-actin binding site which is masked in the intact molecule by the intramolecular head-tail interaction. Moreover, the F-actin cross-linking and stabilizing activities of the tail domain represent novel functions for vinculin that may be important to its physiological role at sites where microfilament bundles attach to the membrane to create a force-transducing linkage. Based on these findings, we propose that assembly of vinculin into an adherens junction involves disruption of the head-tail interaction, revealing sites that mediate high affinity binding to other junctional components. This "opening" or "activation" of vinculin probably comes about through the action of signalling cascades induced by ligation of cell adhesion molecules such as integrin. Identification of the signal transducer(s) involved in activation of vinculin is likely to yield substantial insight into how the signals elicited by cell adhesion molecules result in assembly and disassembly of transmembrane force-transducing structures.

A STABLE MICROFILAMENT-ASSOCIATED SIGNAL TRANSDUCTION PARTICLE IN MAMMARY TUMOR MICROVILLI: PRESENCE OF P185^{neu}, MAP KINASE PATHWAY COMPONENTS AND E-CADHERIN/CATENINS. C.A. Carothers Carraway, M.E. Carvajal, D. Lorenzo and Yongqing Li. Univ. of Miami School of Medicine, Miami, FL 33101

Microvilli purified from sublines of a highly malignant and metastatic subline of 13762 ascites rat mammary adenocarcinoma cells contain a large, microfilament (MF)-linked transmembrane complex (TMC) (1) containing the growth factor receptor p185^{neu} (2). The structural core of the TMC is a large ($> 2 \times 10^6$ Da) complex of at least 5 glycoproteins, Mr's 120, 110, 80, 65 and 55 kDa, in a stable transmembrane association with cytoplasmic actin (3). The TMC-gp's and p185^{neu} purified on Concanavalin A-agarose in 0.05% SDS could be reconstituted into a stoichiometric complex upon displacement of SDS. The large size of the complex and the presence of the growth factor receptor led us to the hypothesis that the TMC-gp's form the nucleus of a signal transduction particle (STP) (2). The present studies report the presence in the STP of other signal transduction components as well as proteins involved in epithelial cell-cell interactions.

Immunoblots showed that the TMC-containing MV fractions and purified TMC contain the components of the recently described growth factor receptor-linked mitogenesis pathway, i.e., GRB2, Sos, Ras, Raf, MAPKK, and MAPK. All of the pathway proteins co-immunoprecipitated with anti-p185^{neu} from extracts of microvilli or MV MF core. Kinase assay of immunoprecipitated MV MAP kinase demonstrated that this key mitogenesis pathway component was constitutively active, whereas alkaline phosphatase-treated microvillar lysates had minimal activity. Recently we have shown that E-cadherin as well as α -, β - and γ -catenins are also associated with the TMC. We envision four important functions for the transmembrane complex. First, it provides a site for interaction with a growth factor receptor p185^{neu}. Second, the p185-containing TMC provides a site for concentrating regulatory elements of the pathway(s) responsible for amplifying and transducing signal from p185^{neu}. This concentration can facilitate "crosstalk" among different elements or pathways of signal transduction cascades. Third, the TMC sequesters E-cadherin/catenin complexes, preventing polarization of the epithelial tumor cells and disrupting cell-cell interactions. Fourth, the TMC serves as a link to microfilaments for both the signal transduction particle (reviewed in ref. 4) and cadherin-catenin complex. We propose that sequestration of the E-cadherin-catenin complex is an important mechanism for permitting dissociation of the tumor cells from tissue, allowing for tumor progression.

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IDENTIFICATION OF ACTIN, VINCULIN, AND INTEGRIN BINDING DOMAINS IN TENSIN SUGGESTS NEW MECHANISMS FOR ACTIN-MEMBRANE ASSOCIATION.

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Tensin, a 215 kD actin capping protein, is localized to a variety of adherens junctions, including focal adhesions of fibroblasts. Phosphorylation of the protein upon cell adhesion to fibronectin and the presence of an SH2 domain in its structure suggest that this actin binding protein is an integrator of cytoskeletal functions and signal transduction. By testing the activity of bacterial fusion proteins containing different segments of chicken cardiac tensin, our laboratory has determined that a high-affinity actin capping domain is within residues S1061-H1145 (Chuang et al., *J. Cell Biol.*, 1995, in press). To further understand how tensin might act as a link between actin and membrane-associated components, we used a microtiter plate assay to study the binding of tensin fusion proteins to vinculin and to integrin. In the first set of experiments, we found that tensin bound to chicken gizzard vinculin with a K_d of 5×10^{-7} M. Binding was saturable and was eliminated by denaturation of tensin. The interaction between the two proteins was also confirmed with blot-overlay and immunoprecipitation assays. By testing the activity of fusion proteins containing different segments of tensin, we established that the vinculin binding site is within residues N790-K1060. In the second set of experiments, we detected saturable binding of tensin to chicken gizzard integrin. Assays on a synthetic peptide with the sequence of the cytoplasmic domain of $\beta 1$ integrin indicated that this region contains the tensin binding site. Measurement of the activity of tensin fusion proteins with different deletions allowed us to narrow the site of integrin binding in tensin to within residues S1061-Y1521. The results of these two sets of experiments suggest that tensin can be a direct link between the barbed ends of actin filaments and integrin. In addition, tensin can also link F-actin to a complex of proteins that includes vinculin, talin, and integrin. How the binding of phosphotyrosine-containing proteins to the SH2 domain and the phosphorylation of tensin might affect its binding to actin, vinculin, and integrin is an intriguing question for future studies. (Supported by NIH grant GM22289)

SITE-SPECIFIC PHOSPHORYLATION AND LOCALIZATION OF MOESIN IN THROMBIN-ACTIVATED HUMAN PLATELETS. Fumihiko Nakamura, Manuel R. Amieva and Heinz Furthmayr. Laboratory of Experimental Oncology, Department of Pathology, Stanford University Medical Center, Stanford, California 94305-5324.

When activated with thrombin, platelets change shape, aggregate and release potent mediators. We have investigated the role of moesin during platelet activation since, of several closely related membrane-cytoskeletal linking proteins, human platelets contain only moesin. Within seconds after exposure to thrombin moesin phosphorylation increases by 160% and returns to baseline levels over a five minute period. This modification affects a single threonine residue in the C-terminal F-actin binding domain. Addition of inhibitors of phosphokinases (e.g. staurosporin) produces a shift to a completely de-phosphorylated form, while phosphatase inhibitors, such as calyculin A, result in complete phosphorylation, as shown by two-dimensional electrophoresis. Normal platelets, activated by contact with glass, spread by sending out filopodia and by flat lamellopodial protrusions to finally assume a pancake-like shape. Moesin is localized in filopodia and throughout the platelet. In contrast, thrombin activation of staurosporine- or calyculin A-treated cells results in platelets with extremely long filopodia. Such cells are incapable of spreading. In spite of similar shapes, the distribution of moesin in platelets treated with kinase or phosphatase inhibitors differs. While moesin in staurosporine-treated cells is found in filopodia and cell bodies co-distributed with actin filaments, in calyculin A cells, unlike actin, it is absent from filopodial protrusions. These data suggest that moesin is not necessarily related to the formation of filopodia, but that phosphorylation plays a role regulating moesin's interaction with filopodial membrane sites and/or actin filaments.

**INTERACTIONS BETWEEN THE *DROSOPHILA lethal(2)giant larvae*
TUMOUR SUPPRESSOR PROTEIN, p127, AND NONMUSCLE MYOSIN II
HEAVY CHAIN**

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Inactivation of the *l(2)gl* gene leads to neoplasia in the optic centers of the larval brain as well as tumours in imaginal discs. The *l(2)gl* gene encodes a 127 kDa protein with no sequence homologies suggesting a possible function.

Using cell fractionation and biochemical procedures as well as histochemistry, we have shown that p127 is a component of a cytoskeletal network extending in the cytoplasm and/or underlying the inner face of the plasma membrane in a variety of cells and tissues. On the membrane, a significant portion of p127 is resistant to non-ionic detergent extraction, a characteristic of components associated with the cytoskeletal matrix (Strand et al., JCB 127: 1361-1373, 1345-1360, 1994). Gel filtration and native PAGE analysis indicated that p127 forms high molecular complexes, resulting essentially from its homo-oligomerization. Binding of p127 to itself was confirmed by blot overlay assays. Three domains of homo-oligomerization, each of about 40 to 50 amino acids in length, were identified in p127. These domains were mapped by fusing portions of p127 to protein A (behaving as a monomer) and testing each fusion protein for its ability to oligomerize by gel filtration analysis and in blot overlays.

To identify other components in p127-complexes, we purified a series of proteins by immuno- and p127-affinity chromatography. Micro-sequencing was performed on two proteins. One of them of a mol. weight of ~48 kDa is of unknown identity whereas the other protein was identified as nonmuscle myosin II heavy chain. Blot overlays on bacterially expressed segments of myosin II have allowed us to map the binding site(s) of p127 to a discrete domain in the tail region of myosin II between a.a. residues 1715 and 1865.

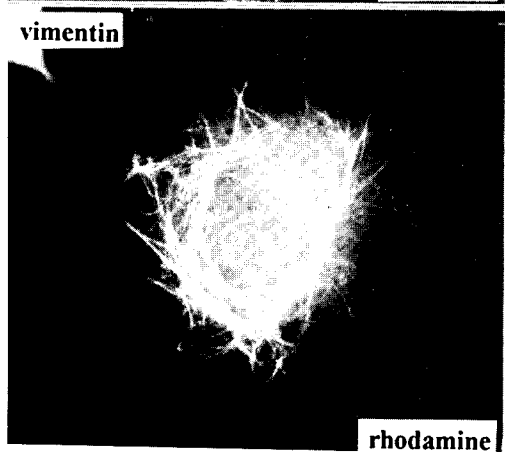
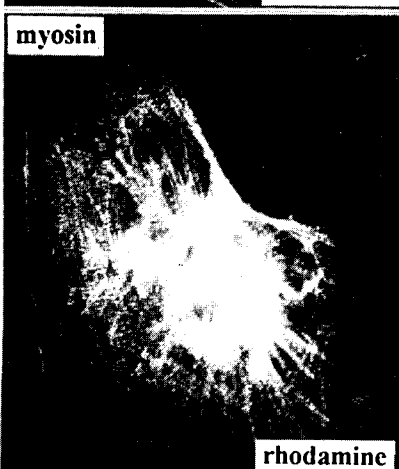
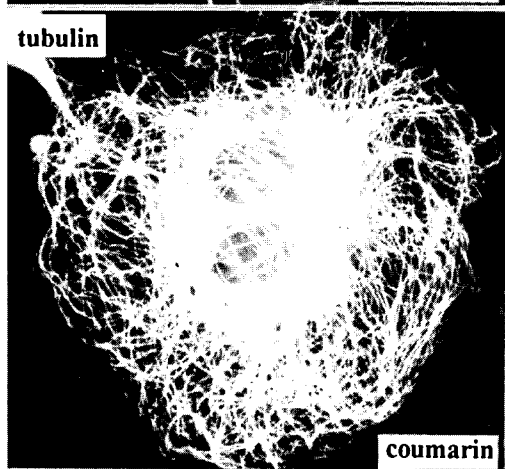
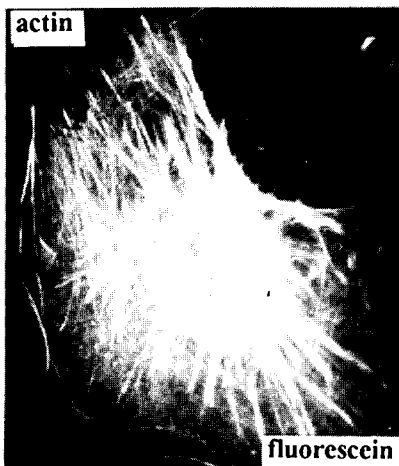
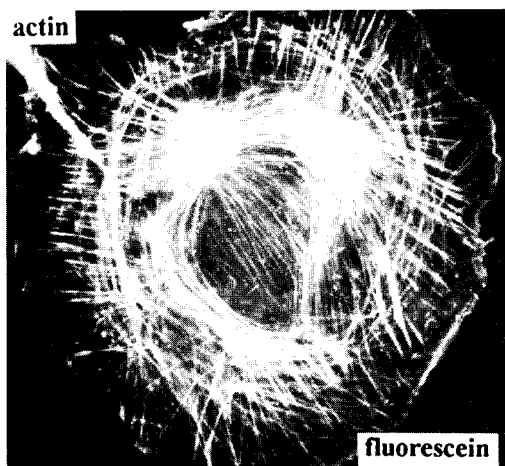
Among the other components of p127-complexes, we biochemically characterized a protein kinase which specifically phosphorylates p127 on serine residues. At present the identity of this kinase remains unknown. However, biochemical investigations showed that p127 is released from purified membranes upon addition of ATP suggesting that the kinase present in p127-complexes may regulate p127 membrane association or complex formation.

All together these results showed that p127 is a component of a cytoskeletal network involving nonmuscle myosin II and is strongly associated with a serine kinase. During *Drosophila* development, the absence of p127 results in the neoplastic transformation of a specific set of the adult anlagen. In the case of the tumorous imaginal disc cells which, during larval development, lose their normal polarity and grow out of the original epithelial monolayer, our biochemical data support a role for p127 in directly maintaining cell shape. In addition the absence of p127 may also alter the domains of cell contacts, preventing the tumorous cells from recognizing their neighbors.

THE CYTOSKELETAL PLAQUE PROTEIN PLAKOGLOBIN
SUPPRESSES THE TUMORIGENICITY OF CELLS FROM MOUSE
AND HUMAN ORIGIN

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The physical integration of cells into functional tissues is achieved through adhesion between cells at cell-cell junctions known as desmosomes and *adherens junctions*. These junctional complexes consist of transmembrane receptors of the cadherin family and, at the cytoplasmic side, of plaque proteins that link these transmembrane receptors to either intermediate filaments or microfilaments, respectively. Plakoglobin is a unique cytoplasmic plaque protein localized in both types of junctions. It is closely related to the *Drosophila* segment polarity gene *armadillo* which was demonstrated to function in the signal transduction pathway driven by *wingless* (*wg*), *wnt* (in *Xenopus*), or *int* (in the mouse). To study the signals conveyed by plakoglobin in mammalian cells, tumor cells from mouse and human, which do not express plakoglobin, were transfected with plakoglobin cDNA and its effect on the phenotype of these cells was examined. Highly malignant SV40-transformed mouse 3T3 (SVT2) cells, stably expressing different levels of plakoglobin, were isolated. Immunofluorescence analysis revealed that the transfected plakoglobin was enriched in areas of cell-cell junctions. This implies that the components necessary for its association with the junctional area were present. When injected into syngeneic mice, there was a very good correlation between the ability to suppress the tumorigenicity of these cells and the level of plakoglobin expressed. Fourteen clones expressing different levels of plakoglobin were isolated from a malignant human renal carcinoma cell line after transfection with plakoglobin. The tumorigenicity of these clones was analyzed in nude mice. The clone expressing plakoglobin at a level similar to that of nontransformed epithelial cells was completely suppressed in its ability to form tumors in nude mice, while with the other clones, tumorigenicity was inhibited in direct correlation with the amount of plakoglobin expressed. The cooperation between plakoglobin and the cadherin receptors in tumor suppression was also investigated. SVT2 cells transfected with N-cadherin showed an increase in the level of the plaque proteins α and β -catenin and a more epithelial organization of the cell culture, but their tumorigenicity in mice was not affected. Transfection of both N-cadherin and plakoglobin into SVT2 cells produced cells that were less tumorigenic than with plakoglobin alone. The results suggest that plakoglobin plays a key role in signals mediated by cell adhesion that control cell growth, and that reconstitution of the cadherin-catenin-plakoglobin complex is an important part of this signaling pathway.



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